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The T cell receptor repertoires of immunodominant CD8 T cell responses to *Theileria parva*

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I declare that the work presented in this thesis is my own original work, except where specified, and it does not include work forming part of a thesis presented successfully for a degree in this or another university.

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Abstract

Previous research has provided evidence that CD8 T cells mediate immunity against infection with *Theileria parva*. However, the immunity induced by one parasite strain doesn't give complete protection against other strains and this is associated with parasite strain specificity of the CD8 T cell responses. There is evidence that such strain specificity is a consequence of the CD8 T cell responses of individual animals being focused on a limited number of immunodominant polymorphic peptide-MHC determinants. Dominant responses to the Tp2 antigen have been demonstrated in animals homozygous for the A10 MHC haplotype. Three Tp2 epitopes recognised by A10+ animals ($Tp2_{49-59}$, $Tp2_{50-59}$ and $Tp2_{98-106}$) have been defined. This project set out to investigate the dominance of these epitopes and to examine the T cell receptor (TCR) repertoires of the responding T cells.

The specific objectives were to: (i) Determine the dominance hierarchies of the three defined Tp2 epitopes in both A10-homozygous and -heterozygous cattle. (ii) Examine the clonal repertoires of epitope-specific responses by analysis of TCR gene expression. (iii) Isolate full-length cDNAs encoding TCR α and β chain pairs from T cell clones of defined epitope specificity and use them to generate cells expressing the functional TCRs. Using MHC class I tetramers the relative dominance of CD8 T cell responses were found to differ between A10-homozygous and heterozygous cattle. All A10-homozygous cattle examined had detectable responses to all 3 Tp2 epitopes, the $Tp2_{49-59}$ epitope consistently being the most dominant. By contrast, only some A10-heterozygous cattle had detectable responses to Tp2 and when present the response was specific only for the $Tp2_{98-106}$ epitope. Analyses of the sequences of expressed TCR β chains showed that the responses in individual animals were clonotypically diverse, but often contained a few large expanded clonotypes. The TCRs of $Tp2_{98-106}$–specific T cells showed preferential usage of the Vβ13.5 gene and the frequent presence of a "LGG" motif within the CDR3 of the B chain. A conserved (public) TCRβ clonotype shared by the $Tp2_{50-59}$-specific CD8 T cells from all A10-homozygous cattle was identified. The TCRα chains co-expressed with this public TCRβ clonotype were identified for a number of T cell clones. Lentivirus transduction of Jurkat cells with three full-length TCR α and β chain pairs resulted in

successful expression of one of the α/β chain pairs as a functional TCR, thus providing the basis for future work to generate bovine T cells expressing defined TCRs *in vitro*.

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Abbreviations

AMV-RT	Avian Myeloblastoma virus reverse transcriptase
bp	base pair
BSA	Bovine serum albumin
°C	Degrees celsius
C	Constant gene segment
Cβ	T cell receptor beta chain constant gene segment
CD	Cluster of differentiation
CDR	complementary determining region
CDR3β	T cell receptor beta chain complementary determining region three
cDNA	Complementarity deoxyribonucleic acid
CO_2	carbon dioxide
D	diversity gene segment
Dβ	T cell receptor beta chain diversity gene segment
dATP	2'-deoxyadenosine triphosphate
dCTP	2'-deoxycytidine triphosphate
dGTP	2'-deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
dNTP	Mixture of dATP, dCTP, dGTP, dTTP
dTTP	2' deoxythymidine triphosphate
EBV	Epstein-Barr virus
ECF	East coast fever
EDTA	Ethylene-diamine-tetra-acetic acid
ELISPOT	enzyme-linked immunosorbent spot
ER	endoplasmic reticulum
ERAP	endoplasmic reticulum aminopeptidase
FACS	Fluorescent-activated cell sorting
FBS	Foetal calf serum
FITC	fluorescent isothiocyanate
g	grams
HCl	hydrochloric acid
HCMV	Human cytomegalovirus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSV	Herpes simplex virus
HTS	High throughput sequencing
IFN	Interferon
IL	interleukin
ILRAD	International Laboratory of Research on Animal Diseases
IPTG	Isopropyl β-D-1-thiogalactopyranoside

J	Joining gene segment
Jβ	T cell receptor beta chain joining gene segment
Kbp	kilo base pairs
LB	Luria-Bertani
LCMV	Lymphocytic Choriomeningitis Virus
mAbs	Monoclonal Antibodies
MACS	Magnetic-activated cell sorting
Mbp	Mega base pairs
µg	micro gram
MHC	Major histocompatibility complex
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
min	minutes
ml	mili litres
µl	micro litres
mM	mili molar
mRNA	messenger ribonucleic acid
ng	nano grams
NK	Natural killer
PBMC	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pCMV	packaging plasmid containing cytomegalovirus promoters
PCR	Polymerase chain reaction
PE	Phycoerythrin
pMD2.G	packaging plasmid containing Vesicular stomatitis virus G protein
pMHC I	peptide-major histocompatibility complex class I ligand
pmol	pico molar
RACE	rapid amplification of cDNA ends
RAG	recombination-activating genes
RNA	ribonucleic acid
RNase	ribonuclease
rIL2	recombinant human interleukin two
rpm	revolutions perminute
RPMI	Roswell Park Memorial Institute medium
RSS	recombination signal sequence
RT	reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction
s	seconds
SCID	severe combined immunodeficiency
SCM	standard culture medium
SMART	switching mechanism at the 5'-terminus of the RNA transcript
TA	*Theileria annulata*-infected cell line
TAE	Tris-acetate-EDTA buffer

TAP	Transporter associated with antigen processing
TCR	T cell receptor
TCRβ	T cell receptor beta chain
Tp	*Theileria parva* antigen
TpM	*Theileria parva* (Muguga)- infected cell
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol
V	Variable gene segment
Vβ	T cell receptor beta chain variable gene segment
v/v	volume to volume
VSV-g	vesicular stomatitis virus glycoprotein
w/v	weight to volume
WHO-IUIS	World Health Organisation-International Union of Immunological Societies
x g	force of gravity

Chapter 1: General introduction

1.1 *Theileria parva*

Theileria parva (*T. parva*) belongs to the *Theileria* genus of apicomplexa protozoa and is the causal agent of an acute and usually fatal lymphoproliferative disease in cattle known as East Coast fever (ECF). This disease is endemic in eastern, central and southern Africa (Figure 1.1) and causes economic losses, which were estimated over 300 million dollars annually [Gachohi et al., 2012]. The parasite is transmitted predominantly by the tick vector *Rhipicephalus appendiculatus*. Another closely related species, *R. zambesiensis,* also serves as vector in some parts of southern Africa [Lawrence et al., 1983, Purnell et al., 1974]. Climate conditions that influence survival and multiplication of the tick vectors influence the distribution and seasonal occurrence of the disease [Norval et al., 1991]. European *Bos taurus* breeds of cattle are highly susceptible to the disease, with mortality as high as 100% in the absence of control methods. Some indigenous breeds of *Bos indicus* cattle show a degree of resistance to *T. parva* infection. However, high level of experimental challenge can overcome the resistance and cause severe fatality in these animals [Ndungu et al., 2005]. The high morbidity and mortality of *T. parva* infections seriously constrain cattle production in areas of Africa where the parasite is present and highlight the need for effective control methods.

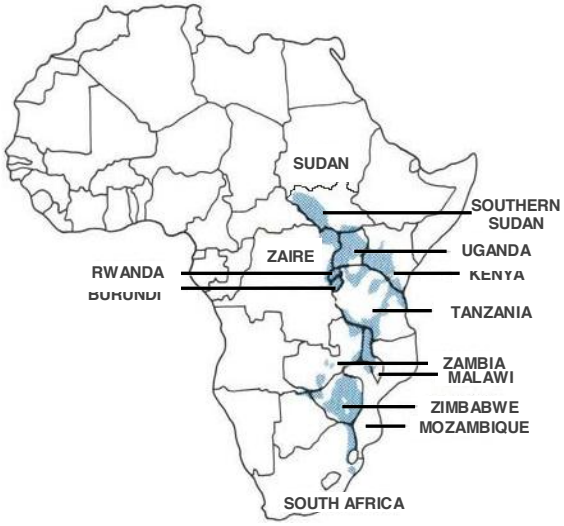


Figure 1.1 Geographic distribution of *T. parva* in Africa. Reproduced from the Annual Report of the International Laboratory of Research on Animal Diseases (ILRAD) 1990

1.1.1 Life cycle of *T. parva*

The life cycle of *T. parva* involves a series of intracellular developmental stages both in the tick vector *R. appendiculatus* and the mammalian host, as shown in Figure 1.2.

After ingestion by nymphal ticks, the intra-erythrocytic piroplasms of *T. parva* differentiate into micro- and macro-gametes which fuse to form zygotes in the gut of the tick. Unlike all stages of development in the mammalian host, this stage has a diploid genome. Parasites derived from zygotes formed by two genotypically different *T. parva* parasites undergo recombination at this stage [McKeever, 2006]. The zygotes invade the gut epithelial cells and differentiate into kinetes which migrate to the salivary gland, where they invade a specific cell type known as the E cell, in which they undergo multiple nuclear divisions. During this process, known as sporogony, a meiotic division occurs resulting in a haploid genome. This process results in production of large numbers of sporozoites by each infected salivary gland cell [Fawcett et al., 1982].

Sporogony and production of sporozoites is not completed until the adult stage of the tick has fed for 3-4 days. Sporozoites are released into the saliva of the feeding

ticks and invade different lineages of lymphocytes of the mammalian hosts. Studies of cell tropisms of *T. parva* infected cells have shown that the vast majority of infected cells in infected cattle are CD4 and CD8 T lymphocytes, although both B and T lymphocytes can be infected with similar efficiency *in vitro* [Morrison et al., 1996, Baldwin et al., 1988]. Unlike other apicomplexan protozoa, which reside in endocytic vacuoles, *T. parva* parasites enter the cytoplasm of host cells through dissolution of host endosomal cell membrane and associate with the host cell microtubules [Shaw, 2003, Shaw et al., 1991]. Within three days, sporozoites develop into multi-nuclear schizonts and induce host cell transformation and proliferation, with parasite division occurring simultaneously with host cell division. The mechanisms of parasite-induced host cell transformation have been explored extensively. Although the way in which the parasite mediates transformation remains unclear, research has indicated that up-regulation of signalling pathways involved in cell proliferation and down-regulation of apoptotic pathways result in continuous multiplication of parasitized host cells [Dobbelaere et al., 2000, Heussler et al., 2006]. A recent study carried out by Marsolier, J. et al. demonstrated that a peptidyl-prolyl isomerase secreted by *T. parva* is capable of degrading host ubiquitin ligase and stabilizing cell proliferation pathways to facilitate cell transformation [Marsolier et al., 2015]. In infected cattle, schizont-infected lymphocytes are detectable in the lymph node draining the site of infection 5-8 days after infection and their numbers increase rapidly over the subsequent 7-10 days. This rapid multiplication of schizont-infected cells is responsible for most of the pathology of the disease. The infected cells disseminate throughout the lymphoid system and also invade non-lymphoid tissues including the lungs and gastro-intestinal tract. In fully susceptible breeds, death usually occurs within 3 weeks of infection, mainly due to respiratory distress as a consequence of pulmonary oedema [Irvin et al., 1983]. In the later stages of infection, a proportion of schizonts differentiate to merozoites, which, upon release, invade erythrocytes giving rise to the tick-infective piroplasm stage. Small numbers of both schizonts and piroplasms persist in recovered animals for months or years; the infected cells are usually not observed microscopically but can be detected by PCR [Skilton et al., 2002]. Tick transmission from animals with persistent infection

is believed to be the predominant source of infection for cattle in ECF-endemic areas [Medley et al., 1993].

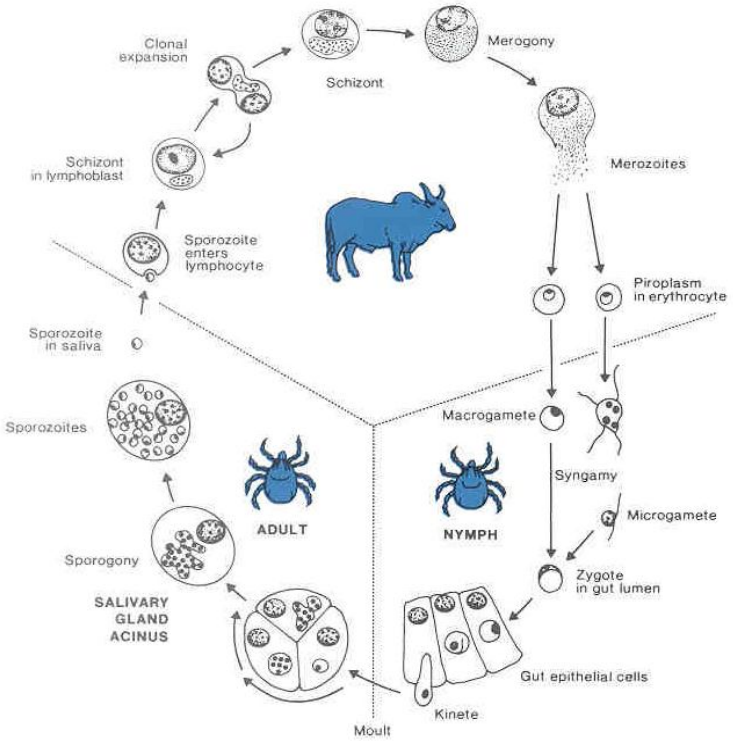


Figure 1.2 The life cycle of *T. parva*. Reproduced from the Annual Report of the International Laboratory of Research on Animal Diseases (ILRAD) 1990.

1.1.2 Control of *T. parva*

Theilericidal compounds were introduced for treatment of theileriosis in the 1980s. The most effective compound is buparvaquone [McHardy et al., 1985]. However, treatment needs to be applied relatively early in the disease to be effective, thus requiring early diagnosis. Treatment in the later stages can sometimes be effective but may require repeat treatment and recovered animals have reduced productivity. Chemotherapy is also expensive. Beside theilericidal treatment, regular application of acaricides to prevent tick infestation has been used to control the disease. However, this method also has shortcomings, namely the need for continuous application, the emergence of drug resistant tick populations and

concerns about chemical contamination of the environment [Morrison and McKeever, 2006].

Vaccination is considered to be a more sustainable way of controlling the disease, but current vaccines also have limitations [McKeever, 2007, Morrison and McKeever, 2006].

1.1.2.1 Live parasite vaccination

Since animals recovered from clinical disease are immune to subsequent challenge, early studies attempted to immunise cattle with materials derived from tissues of sick or recovered animals, including blood and cell suspensions of spleen and lymph node. However, this method sometimes resulted in disease and only about 70% of surviving animals were protected against subsequent field challenge [Spreull, 1914]. The failure to achieve full protection was later demonstrated to be due in some instances the insufficient numbers of infected cells administered and also the differences of major histocompatibility complex (MHC) genotypes between the donor cells and the recipient animals [Pirie et al., 1970, Buscher et al., 1984]. The development of *in vitro* culture of parasitized cells and cryopreservation of parasites in the 1960s and 1970s made it possible to produce live vaccines using standardised doses of parasites [Brown et al., 1973, Cunningham et al., 1973]. A method of infection and treatment was developed, which involves simultaneous administration of long-acting oxytetracycline and a defined dose of cryopreserved sporozoites. Animals immunised by this method show solid immunity against homologous parasite strains but variable protection against heterologous strains [Radley et al., 1975a]. Because of this immunological diversity, various stocks of *T. parva* were tested for their ability to induce immunity against other stocks. A mixture of 3 stocks, known as the 'Muguga cocktail' (containing the Serengeti-transformed, Kiambu 5 and Muguga stocks) was found to provide wider protection than each of the individual stocks [Radley et al., 1975b, Radley et al., 1975a]. This Muguga cocktail has been used successfully for vaccinating cattle in the field in some areas of Africa [Marcotty et al., 2002, Di Giulio et al., 2009]. Two batches of the Muguga cocktail vaccines, produced in 1996 and 2008 respectively by ILRI, were proved to provide

95-100% protection against ECF. With the safety and effectiveness of the later batch during field trials in Kenya, it was launched in 2012 for national distribution [Gachohi et al., 2012]. However, it has a number of practical disadvantages, including the requirement of a cold chain for preserving and transporting vaccine stocks and the perceived threat of introducing the vaccine parasite strains into local tick populations [Uilenberg, 1999]. These shortcomings have limited the widespread use of this vaccination approach.

1.1.2.2 Approaches to subunit vaccine development

To overcome these obstacles, research has been focusing on analyses of the immune response to identify immunogenic parasite antigens that can induce protective humoral or cellular immune responses for vaccination.

Although infection and treatment induces little or no antibody against the sporozoite stage, immunisation with a 67-kDa sporozoite surface protein (termed p67) was found to induce antibody that neutralised the infectivity of sporozoites *in vitro* [Musoke et al., 1984]. Experiments were carried out to investigate the protective efficiency of vaccination against the sporozoite stage of parasite. Experimental immunisation of cattle with recombinant p67 in adjuvant resulted in approximately 50% protection against challenge with a lethal dose LD_{70} dose of cryopreserved sporozoites, but lower levels of protection were achieved after natural challenge [Musoke et al., 2005, Musoke et al., 1992]. No obvious correlation between the isotype or level of antibody induced and immunity to *T. parva* was obtained. In terms of epitope specificity, antibody responses showed no differences between protected and susceptible animals [Nene et al., 1999]. Moreover, immunization of cattle with recombinant p67 antigen produced in different expression systems (prokaryotic or eukaryotic) resulted in similar levels of protection [Kaba et al., 2005, Bishop et al., 2003]. Subsequent studies of the MHC genotypes of vaccinated animals suggested that MHC class II (MHC II) genotype could influence the efficacy of protection provided by p67 vaccination [Ballingall et al., 2004].

The essential role of cell-mediated immunity against *T. parva* infection was observed by demonstrating protection following transfer of lymphocytes from

immune to naïve twin calves. The recipient animals receiving transferred cells in the period immediately following parasite challenge were able to eliminate parasite infection and subsequently showed solid immunity to challenge with a lethal dose of *T. parva* [Emery, 1981].

The ability to establish *T. parva*-infected cell lines *in vitro* has greatly facilitated the investigation of parasite-specific T cell responses [Brown et al., 1973]. These cells have been demonstrated to be effective antigen-presenting cells for *in vitro* activation of *T. parva*-specific memory T cells from the blood of immune cattle [Pearson et al., 1979]. Responses by both CD4 and CD8 T cells specific for parasitized cells can be generated from cattle immunized with *T. parva* [Baldwin et al., 1987, Goddeeris et al., 1986] and these cells can be maintained as T cell lines by repeated antigenic stimulation and cloned, enabling detailed analyses of parasite-specific T cell responses. The CD8 T cell lines show MHC class I (MHC I) - restricted cytotoxic activity against *T. parva*-infected cells and similar cytotoxic activity is detected in PBMC assayed *ex vivo* from cattle undergoing immunisation or challenge [Morrison et al., 1987, Goddeeris et al., 1986]. Adoptive transfer of CD8 T cell enriched populations from immune to naïve twin calves following parasite challenge of the naïve twin was found to confer protection, providing clear evidence of an important role of the CD8 T cell response in immunity against *T. parva* [McKeever et al., 1994]. These results prompted subsequent studies to identify immunogenic antigens recognized by CD8 T cells for vaccine development. Several *T. parva* antigens were identified and used for immunization of cattle to test their vaccination potential. Using a prime-boost protocol, involving priming with recombinant plasmid DNA or canarypox viruses expressing 5 *T. parva* antigens and a single boost with recombinant vaccinia virus Ankara strain, most of the immunised animals were found to generate specific CD8 T cell responses, detected with an IFNγ ELISPOT assay, but less than 50% of them (10/24) survived parasite challenge [Graham et al., 2006]. These results suggested that the protective immune responses against *T. parva* are complex and that further understanding of T cell mediated immune responses against the parasite would be informative for effective vaccine design.

1.1.3 T cell mediated immunity against *T. parva*

As discussed above, there is strong evidence that CD8 T cell responses play an important role in immunity to *T. parva*. However, CD4 T cells from immune cattle also show strong parasite-specific proliferative responses and results from cell mixing experiments *in vitro* suggest that they may provide help for CD8 T cell activation [Taracha et al., 1997, Baldwin et al., 1987, Baldwin et al., 1992].

1.1.3.1 Strain specificity of CD8 T cell mediated immune responses against *T. parva*

Parasite strain specificity of immunity to *T. parva* was observed when animals were immunized with one parasite strain and subsequently challenged with a second heterologous strain; while some animals were protected, others remained susceptible to the challenge [Radley et al., 1975a, Irvin et al., 1983]. This incomplete cross-protection suggested the differential recognition of parasite strains by the protective immune response. Later studies demonstrated that parasite-specific CD8 T cells generated from animals immunized with one parasite strain showed variable recognition of cells infected with other parasite strains [Morrison et al., 1987, Goddeeris et al., 1986]. Moreover, for animals immunized with the same parasite strain, profiles of strain specificity of the responding CD8 T cells showed variation between individual animals, suggesting a possible influence of MHC I genotype [Goddeeris et al., 1990, Morrison et al., 1987]. The interpretation of these experiments was partly complicated by genotypic and antigenic heterogeneity of some of the parasite isolates used in the studies [Goddeeris et al., 1990]. This problem was later resolved by the production of cloned sporozoite stocks [Morzaria et al., 1995]. Further experiments were carried out to explore the relationship between parasite strain specificity of the CD8 T cell response and immunity to parasite challenge. Using the Muguga strain of *T. parva*, which was antigenically homogeneous, for immunisation followed by a cloned derivative of the Marikebuni stock (Marikebuni 3219) for challenge of the immunised animals, a close correlation was observed between the strain specificity of the CD8 T cell response induced by immunisation and the susceptibility of animals to challenge [Taracha et al., 1995a].

Immunized animals with cross-reactive CD8 T cells were protected against the heterologous parasite challenge, while animals with CD8 T cell responses specific for the immunizing strain showed severe clinical reactions. These results provided further evidence for the direct involvement of CD8 T cell responses in immunity against *T. parva* infection and also indicated that different animals appear to respond to different antigens. Further analyses of the CD8 T cell response in animals that recovered from the severe challenge reactions (with the aid of treatment) showed that they included CD8 T cells that recognised both parasites. The absence of responses to these conserved antigens following the initial immunisation suggested that there is a hierarchy in dominance of antigens capable of generating a CD8 T cell response and based on these findings it was proposed that strain specificity is likely to be a consequence of recognition of a limited number of antigenic determinants by individual animals. The polymorphism of these antigens, together with an effect of MHC I genotype on antigen dominance, would explain both the variable CD8 T cell specificity between animals immunized with the same parasite isolate and the different cross-protection profiles of animals [Taracha et al., 1995a, Morrison et al., 1987].

1.1.3.2 *T. parva* antigens recognized by CD8 T cells

Identification of antigens recognized by CD8 T cells has greatly facilitated studies to elucidate the fine antigenic specificity of CD8 T cell responses against *T. parva*. The complete genome sequence of the *T. parva* Muguga strain provided important information for selection of candidate genes [Gardner et al., 2005]. Just over 4000 annotated genes were predicted to encode proteins, over 60% of which were later shown to have detectable transcripts in *T. parva*-infected lymphoblasts [Bishop et al., 2005]. Six *T. parva* antigens recognized by parasite-specific CD8 T cells were initially identified using two parallel approaches, both involving co-transfection of cells with MHC I heavy chain cDNA along with parasite cDNA and screening for recognition of the transfected cells by parasite-specific CD8 T cell lines, using the measurement of interferon-γ (IFN γ) production [Graham et al., 2006]. The first approach used a series of pools, each containing 50 parasite cDNAs from a schizont cDNA library, while the second used single candidate cDNA of genes with

predicted signal peptides which indicate potential secretion into the host cell cytoplasm [Nacer et al., 2001]. For cDNA pools with positive responses, further testing was carried out to define the cDNA recognized by CD8 T cells. This antigen screening approach was adapted from a human system, where tumor antigens recognized by specific CD8 T cells were successfully identified [De Plaen et al., 1997]. Of the six identified antigens, Tp4, Tp5, Tp7 and Tp8 were annotated to encode proteins with functional orthologues in other organisms, while Tp1 and Tp2 were annotated as hypothetical proteins [Graham et al., 2006, Gardner et al., 2005]. Subsequent studies involving analysis of the sequences of the genes encoding Tp1 and Tp2 in different parasite isolates demonstrated that these antigens are highly polymorphic and that CD8 T cells specific for them show parasite strain restriction [Pelle et al., 2011, MacHugh et al., 2009]. Of the 82 analyzed *T. parva* isolates, a number of 42 protein variants were observed for Tp2 antigen [Pelle et al., 2011].

1.1.3.3 Immunodominance of parasite-specific CD8 T cell responses

It has been proposed that parasite strain specificity of bovine CD8 T cell responses against *T. parva* was likely to be a result of dominant recognition of a limited number of peptide-MHC I (pMHC I) determinants by the responding CD8 T cells [Taracha et al., 1995b], a phenomenon that has been defined as immunodominance (see section 1.3). The identification of CD8 T cell antigens enabled further investigation of the antigen specificity of CD8 T cell responses. Studies of CD8 T cells from animals of defined MHC I genotypes known to recognize the six antigens identified nine epitopes together with their MHC I restriction elements [Graham et al., 2008]. Cattle expressing the BoLA-A18 (A18) and BoLA-A10 (A10) MHC I haplotypes were found to respond strongly to the Tp1 and Tp2 antigens respectively [Graham et al., 2006, Graham et al., 2007, MacHugh et al., 2009]. In a study where cattle with defined BoLA haplotypes were immunized with respective *T. parva* antigens, ~50% of animals showed dominant responses to the antigens used for immunization. For cattle with A10 haplotype, CD8 T cell responses to the Tp2 antigen were found absent in some animals, which was suggested due to a lack of potency of vaccination protocols [Graham et al., 2008]. Two epitopes in the Tp2 antigen, $Tp2_{49\text{-}59}$ and $Tp2_{98\text{-}106}$, were confirmed to be

restricted by the MHC I allele 2*01201 and in two immune A10-homozygous animals >60% of the responding CD8 T cells were specific for the $Tp2_{49-59}$ epitope with a small component of CD8 T cells specific for the $Tp2_{98-106}$ epitope [MacHugh et al., 2009]. These animals did not have detectable responses to 3 further antigens (Tp4, Tp5 and Tp8) examined in these experiments. These results supported the hypothesis that strain specificity of CD8 T cell responses against *T. parva* was associated with immunodominant responses against certain *T. parva* antigen in individual animals and that the antigen dominance is determined by MHC I genotype. The findings also suggested a strong hierarchy in dominance among the antigenic determinants that elicit a response in an animal of a particular MHC I type [MacHugh et al., 2009].

1.1.3.4 CD8 T cell protection after *T. parva* antigen immunization

Although the attempts to immunise cattle with recombinant *T. parva* antigens using viral vectors did not result in protection, most animals mounted a specific CD8 T cell response. However, only in a few of these animals did the CD8 T cells show cytotoxic activity against autologous parasitized cells [Graham et al., 2006]. Studies of calves undergoing primary infection with *T. parva* following inoculation with a lethal dose of sporozoites demonstrated a potent CD8 T cell response in the draining lymph node coinciding with initial microscopic detection of parasitized cells. However, the responding cells did not show parasite-specific cytolytic activity and did not appear to exert any control of the infection [Houston et al., 2008]. These results suggest that appropriate functional differentiation of the induced antigen-specific CD8 T cells may be a critical factor in determining their protective activity.

1.2 CD8 T cell-mediated immune responses

T cell-mediated immune responses have been demonstrated to be an important component of the vertebrate immune system and in mammals are essential for effective immune responses against many pathogens. They have the ability to recognize and respond to a vast array of different antigens that an individual may encounter during their life time [Kaech et al., 2002]. T cells recognize foreign antigens utilising a T cell receptor (TCR), a heterodimer molecule composed of α

and β chains, expressed on the cell surface [Davis and Bjorkman, 1988]. The recognition of diverse antigens depends on the diversity of the TCR repertoire, which is generated by a series of site-specific recombination events involving several gene segments during the maturation of T cells in the thymus [Schlissel, 2003, Spits, 2002, Lieber, 1991].

Mature αβ T cells are composed of two subpopulations based on expression of different co-receptors, CD4 and CD8 [Germain, 2002]. CD4 T cells, also known as helper T cells, recognize antigenic epitopes generated in the endocytic pathway, most commonly from exogenous proteins, and presented by MHC II molecules on the surface of antigen presenting cells (APCs). CD8 T cells, also referred to as cytolytic T cells (CTL), respond to epitopes derived from endogenously produced antigenic proteins. Such proteins are degraded in the proteasome and peptide products are transported into the endoplasmic reticulum where they associate with newly synthesised MHC I molecules before moving to the surface of infected cells or APCs [Blum et al., 2013]. CD8 T cell-mediated immune responses have been demonstrated to have a critical role in immunity to many viruses, some parasites and bacteria [La Gruta and Turner, 2014, Corradin and Levitskaya, 2014, Nagata and Koide, 2010, Harty and Bevan, 1999]. Understanding the mechanisms by which CD8 T cell responses are induced and how they mediate immunity has important implications for rational design of T cell based vaccines [Pulendran et al., 2013, Hansen et al., 2011, Yewdell, 2010, Kaech et al., 2002].

1.2.1 MHC I-associated antigen presentation

During infection with intracellular pathogens, large numbers of peptides can be generated from pathogen derived antigenic proteins as a result of protein degradation by proteasomes. Peptides with 8-22 amino acids in length [Kisselev et al., 1999] located in the cytoplasm are transported by the transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER) where they are trimmed by aminopeptidases before associating with newly synthesised MHC I heterodimers [Reits et al., 2003, Rock et al., 1994, Serwold et al., 2002]. MHC I proteins possess pockets within the peptide-binding groove, which preferentially bind particular

amino acid residues, usually at position 2 and at the C terminus of the peptide. These pockets differ between MHC I alleles resulting in different amino acid binding preferences [Falk et al., 1991]. Peptides that are suitable for MHC I binding are normally 8-11 residues in length, but occasionally can be longer, up to 14 amino acids [Burrows et al., 2006, Falk et al., 1991]. The MHC I heavy chain, β2 microglobulin and peptide are all required for assembly of the functional MHC I protein. Once assembled, the peptide-MHC I complexes (pMHC I) are transported via the Golgi apparatus to the cell surface. This whole process is referred to as classical MHC I-associated antigen presenting pathway [Yewdell et al., 2003], as shown in Figure 1.3.

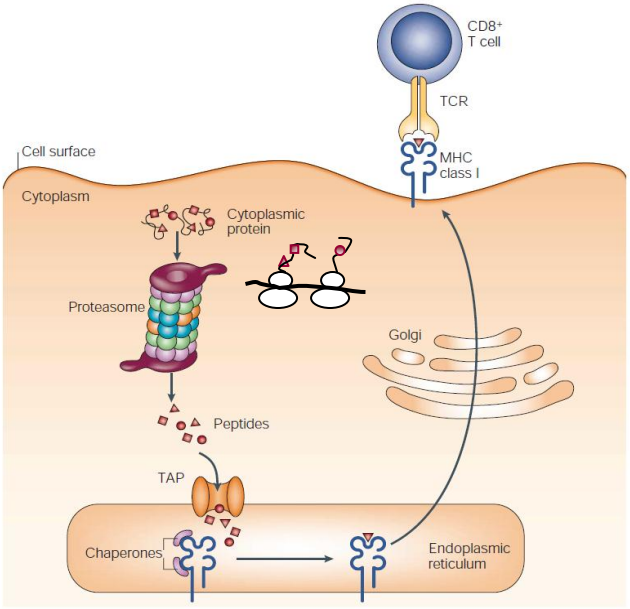


Figure 1.3 Classical MHC I antigen presentation. Endogenous proteins are degraded by proteasome into peptides, which are then transported by the transporter for antigen processing (TAP) into the lumen of endoplasmic reticulum (ER) for MHC I binding. Assembled peptide-MHC I complexes are released and translocated to the surface of infected cells for CD8 T cell recognition. The figure was copied from a review by [Yewdell et al., 2003] under the permission of publisher.

In addition to the classical pathway, MHC I molecules on professional antigen presenting cells (APCs), mainly dendritic cells (DCs), can also process and present antigen from exogenous sources, by a process named as cross-presentation [Nair-Gupta and Blander, 2013, Dolan et al., 2012, Heath and Carbone, 2001a]. Although

the precise mechanisms for cross-presentation are not yet fully understood, this pathway is now believed to be important in induction of CD8 T cell responses, particularly against pathogens that do not infect professional APCs [Joffre et al., 2012].

1.2.2 Mechanism and regulation of TCR α and β chain rearrangement

The genes encoding each chain of αβTCR are generated during T cell commitment in the thymus by a process of somatic recombination from several discontinuous gene segments, as shown in Figure 1.4. The β chain comprises variable (V), diversity (D), joining (J) and constant (C) gene segments, while the α chain comprises V, J and C segments. The diverse repertoire of TCR is produced, firstly, through recombination of different combinations of V-J (α) or V-D-J (β) gene segments, and second by removal and addition of nucleotides at the junctions (referred to as junctional diversity) during the recombination process [Turner et al., 2006].

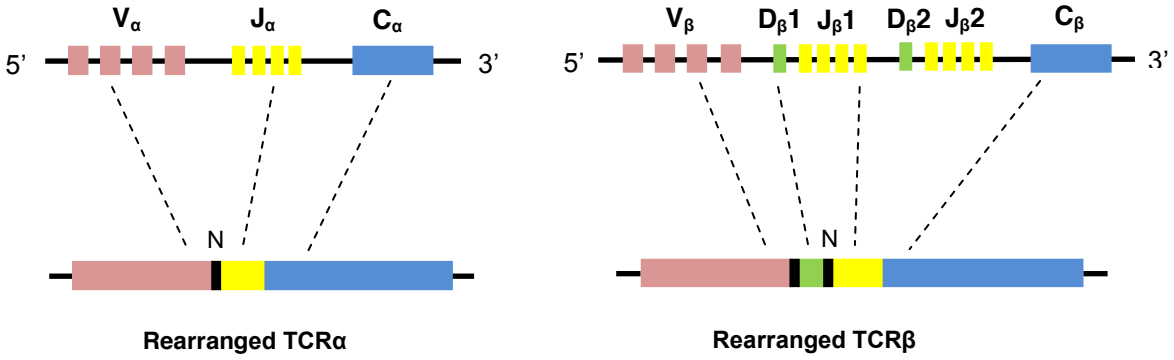


Figure 1.4 Schematic illustration of somatic recombination of TCR α and β genes. Functional αβTCR are generated by somatic gene recombination of variable (V) and joining (J) gene segments for TCRα chain and V, diversity (D) and J gene segments for TCRβ chain. Rearranged gene segments are then combined with constant (C) region to form the functional αβTCR. V, D, J and C gene segments are shown in pink, green, yellow and blue respectively. During somatic recombination, non-germline (N, shown in black) regions are generated due to nucleotide additions and deletions. The figure was re-generated according to [Turner et al., 2006] .

The initiation of gene segment recombination is triggered by the recognition of recombination signal sequences (RSS) by a hetero-multimer formed by proteins

encoded by recombination-activating genes 1 and 2 (RAG1 and RAG2) [Oettinger et al., 1990]. Gene segments of TCR α and β chains are flanked by RSS, which are composed of a conserved heptamer and nonamer separated by a spacer of either 12 (12-RSS) or 23 (23-RSS) nucleotides in length. Different spacer lengths of RSS allows recombination to occur only between a 12-RSS and a 23-RSS, known as the 12/23 rule. This rule prevents undesired rearrangement between different V gene segments and guarantees efficient V(D)J recombination [Bassing et al., 2002]. However, the 12/23 rule is avoided for Vβ-Jβ recombination of the TCR β chain. This mechanism is referred to as the "beyond 12/23 rule" [Drejer-Teel et al., 2007, Olaru et al., 2005, Tillman et al., 2004]. DNA double-strand ends are introduced during V(D)J rearrangement. Joining of these ends is under the control of the ubiquitously expressed non-homologous DNA end-joining (NHEJ) proteins [Kim et al., 2000].

Regulation of gene segment rearrangement is essential to ensure that it only occurs during a specific developmental stage and (in the case of TCRβ) that only a single functional rearranged gene is expressed on the cell surface; rearrangement of TCR α chains permits expression of two α chains, one from each haplotype [Schlissel, 2003, Khor and Sleckman, 2002]. In developing αβ T cells, the TCRβ chain assembles before the TCRα chain. Progenitor cells with a successfully rearranged TCRβ chain co-express a pre-TCRα chain (pre-Tα) which facilitates cell surface expression, allowing selection and survival of those cells with a functional TCRβ chain. Successful rearrangement on one haplotype in most cases prevents rearrangement on the other haplotype, a mechanism known as allelic exclusion, so that only a single TCRβ chain is expressed on each T cell [Khor and Sleckman, 2002, Aifantis et al., 1997]. The rearrangement of TCRα chain occurs only after productive TCRβ rearrangement and is not subject to allelic exclusion; therefore, those T cells that generate in-frame α chains from both haplotypes can express two rearranged TCRα chains [Niederberger et al., 2003, Padovan et al., 1993]. Studies using mouse models have demonstrated that 30% of mature αβT cells express two in-frame TCRα chains [von Boehmer and Melchers, 2010, Brady et al., 2010].

Rearrangement of the TCR occurs in the thymic cortex and during this stage of development the T cells co-express CD4 and CD8. T cells with fully rearranged αβTCRs undergo positive and negative selection in thymus and differentiate into mature CD4 or CD8 single-positive (SP) T cells before entering the peripheral circulation [Starr et al., 2003, Spits, 2002]. During positive selection, which occurs in the thymic cortex, αβT cells expressing TCRs with low or high affinity for self-peptide-MHC complexes expressed on thymic epithelial cells are rescued from apoptosis. The cells then differentiate to CD4 and CD8 single positive T cells in the thymic medulla and undergo negative selection resulting in deletion of those αβT cells expressing TCRs with high-affinity for self-peptide-MHC complexes. This results in a repertoire of mature T cells with appropriate αβTCRs capable of interacting with self-MHC molecules without harmful auto-reactivity [Starr et al., 2003]. Although only a small proportion (2%) of thymocytes survive the selection process, the resultant αβT cells show a very high level of clonal diversity [Nikolich-Zugich et al., 2004].

1.2.3 Diversity of the αβTCR repertoire

1.2.3.1 Quantification of αβTCR diversity

Clonal diversity of the αβTCR repertoire reflects the ability of T cell populations to respond efficiently to a vast array of antigens generated from infections with pathogens [Birnbaum et al., 2012, Turner et al., 2009]. This diversity is a consequence of the effects of somatic V(D)J recombination, including junctional diversity, and subsequent thymic selection based on the interaction of the TCRs with self-peptide-MHC complexes [Vrisekoop et al., 2014, Nikolich-Zugich et al., 2004]. The human genome contains 42 V and 61 J gene segments in the TCRα locus and 47 V, two D, and 13 J segments in the TCRβ locus [Lefranc et al., 1999].

In addition to the random recombination of the different gene segments, non-germline nucleotide additions and deletions during the joining of gene segments substantially increases the diversity of the recombined genes. However, thymic selection markedly limits the diversity, with an estimated reduction of up to 100 fold [Bouneaud et al., 2000, Ignatowicz et al., 1996]. A theoretical diversity of 1×10^{13}

possible TCRs (in the absence of thymic selection) has been calculated, which is greater than the 1×10^{12} T cells present in a single human individual [Arstila et al., 1999]. However, based on experimental data, the actual αβTCR repertoire is estimated to be much lower. Applying the CDR3 spectratyping technique, it was calculated that human naïve T cells contain 2.5×10^{7} αβTCRs [Arstila et al., 1999]. The diversity of the TCR repertoire in memory T cells was estimated as 2×10^{6}.

The diversity of the bovine αβTCR repertoire is yet to be determined. Although the assemblies of the bovine TRA and TRB gene loci in the bovine genome are incomplete, comparison of available cDNA sequences with the genes so far identified in the genome assembly indicates that most variable gene subgroups have been identified [Connelley et al., 2009]. Analysis of the bovine TCRβ chain (TRB) locus has revealed 24 Vβ subgroups including 134 V genes, of which 79 were predicted to be functional. The locus contained 3 clusters of D, J and C genes, each comprising a single TRBD gene, 5-7 TRBJ genes and a single TRBC gene [Connelley et al., 2009]. Genomic analysis of the bovine TCRα locus identified 162 functional V and 52 functional J gene segments within a 3.5Mbp region of chromosome 10 [Connelley et al., 2014]. The massive expansion of Vβ and Vα gene repertoires observed in the bovine genome, compared to humans and mice, has occurred as a result of tandem duplications of DNA regions containing several V gene segments. In the bovine TRB locus, extensive duplication of genes within the TRBV6, 9 and 21 subgroups has resulted in these subgroups accounting for 68% of the identified bovine Vβ genes [Connelley et al., 2014, Connelley et al., 2009]. The increased number of available genes for somatic recombination potentially increases the level of diversity of the bovine αβTCR repertoire.

1.2.3.2 Factors influencing αβTCR diversity

Both the genotype of host MHC and the complexity of self-peptides have been demonstrated to influence the diversity of the mature αβT cell repertoire. Crystal structures of TCR-peptide-MHC complexes show that contact between the TCR and pMHC occurs via 3 loops in each TCR chain, termed complementarity-determining regions (CDR) 1, 2 and 3. CDR1 and CDR2 are encoded within the V gene segments,

whereas CDR3 is encoded by the V-(D)-J junctional region [Bridgeman et al., 2012, Rudolph et al., 2006, Kjer-Nielsen et al., 2003, Garcia et al., 1996]. CDR1 and CDR2 regions of the TCR primarily, but not exclusively, engage the alpha helices of the MHC molecule whereas CDR3 region of the TCR contact with the peptide presented by the MHC molecule [Garcia et al., 2009]. It has been observed that individuals with certain MHC allele showed a degree of bias in usage of V gene segments [Garcia et al., 2009, Sim et al., 1998]. However, αβTCR repertoires in inbred mice with identical genetic background showed limited overlap indicating that generation of the repertoire involves random events and that there is flexibility in the precise make-up of the TCRs capable of responding to any given antigen [Bousso et al., 1998]. The peptide pool participated in thymic selection was illustrated to influence the TCR repertoire composition [Hogquist et al., 1993], which is consistent with the involvement of the CDR3 region in interacting with the MHC-bound peptide [Stewart-Jones et al., 2003]. In a controlled mouse model where a single peptide was used during thymic selection, the resultant naïve T cell repertoire was found to be impaired compared to the repertoires selected by diverse peptides [Barton and Rudensky, 1999].

1.2.4 Naïve antigen-specific T cells

Mature αβ T cells released from thymus make up the naïve repertoire of antigen-specific T cells, which circulate between the blood and lymphatic systems to provide immune surveillance [Boyman et al., 2009, Spits, 2002]. Quantification studies of human and mouse repertoires have provide evidence that the frequency of antigen-specific naïve T cells ranges from 1 to 100 per million [Jenkins and Moon, 2012, Alanio et al., 2010, Obar et al., 2008, Moon et al., 2007]. Upon antigen activation, naïve antigen-specific T cells undergo extensive clonal expansion and differentiation to become effector and memory T cells [Kaech et al., 2002].

Both the quantity and quality of naïve antigen-specific T cell precursors have been investigated to understand their influences on the initiation and maintenance of T cell responses against a given antigen [Turner et al., 2009, Davenport et al., 2007, La Gruta et al., 2006a].

These studies indicate that in some model systems the frequency of naïve T cell precursors can be a predictor of the magnitude of the primary antigen-specific T cell response [Jenkins and Moon, 2012, Davis et al., 2011, Kotturi et al., 2008]. In a lymphocytic choriomeningitis virus (LCMV) mouse model, in which CD8 T cells responding to three dominant epitopes account for a third of the total response, the magnitude of CD8 T cell responses to each of 28 identified epitopes was found to be closely related to the numbers of naïve T cell precursors [Kotturi et al., 2008]. The same phenomenon has also been reported for responses to other viruses, including an experimental model of influenza virus infection in humanized HLA-transgenic mice and humans infected with hepatitis C virus [Tan et al., 2011, Schmidt et al., 2011]. These studies indicated that a large number of naïve T cell precursors is advantageous for generating a T cell response against the respective pathogen epitopes. There is also evidence that the frequency of naïve T cell precursors has an effect on differentiation of the memory T cell populations [Marzo et al., 2005]. Central (T_{CM}) and effector (T_{EM}) memory T cell subsets have been defined according to the expression of homing and chemokine receptors including CD62L and CCR7 [Farber et al., 2014, Masopust et al., 2001]. T_{CM} cells ($CD62L^{+}CCR7^{+}$) preferentially recirculate through secondary lymphoid organs, are long-lived and proliferative strongly in response to antigen challenge, whereas T_{EM} cells ($CD62L^{-}CCR7^{-}$) recirculate through non-lymphoid tissues and mediate rapid responses against re-infection [Sallusto et al., 1999]. In an study involving adoptive transfer of naïve TCR-transgenic T cells, which showed a linear differentiation pathway from naive to T_{EM} to T_{CM} cells, a low-frequency of epitope-specific precursors was found to be associated with failure to differentiate T_{EM} cells into T_{CM} cells, whereas precursors present at high frequency differentiated into both T_{EM} and T_{CM} cells [Marzo et al., 2005].

T cell avidity is a functional parameter, usually measured by determining theminimum concentration of antigenic epitope required to stimulate a T cell response. It reflects the combined effect of several biological variables, including TCR affinity for the peptide-MHC I, the level and type of co-receptor expression and sensitivity to cytokine stimulation, which collectively influence the functional

sensitivity of the T cells [Derby et al., 2001, La Gruta et al., 2006a, Price et al., 2005]. In a SCID (severe combined immunodeficiency) mice model inoculated with human CD8 T cells specific for an immunogenic epitope derived from human immunodeficiency virus (HIV), high-avidity CD8 T cells were found to provide better protection than low-avidity CD8 T cells against challenge with recombinant vaccinia virus expressing epitope-containing HIV antigen [Derby et al., 2001, Alexander-Miller et al., 1996]. High-avidity CD8 T cells were able to respond rapidly and kill early virus-infected cells.

1.2.5 TCR repertoire of antigen-specific CD8 T cell responses

1.2.5.1 Repertoire diversity with immune defense

TCR repertoire diversity has been proposed to influence the magnitude and protective efficacy of antigen-specific CD8 T cell responses [Bousso et al., 1998, Campos-Lima et al., 1997]. The first direct evidence was reported using a mouse model of Herpes simplex virus (HSV) infection, which resulted in a dominant CD8 T cell response to a glycoprotein B-derived peptide (495-502; SSIEFARL) presented by H-2K^{bm8}. The CD8 T cell response showed variable TCR diversity and animals with a diverse repertoire survived virus infection while those with low diversity did not [Messaoudi et al., 2002]. A correlation between the magnitude of CD8 T cell responses and the diversity of the utilized TCR repertoire has also been observed in human HLA-A11-restricted CD8 T cell responses against Epstein-Barr virus (EBV) nuclear antigen-4. CD8 T cells specific for a dominant (EBNA4 416-424) epitope utilised a diverse TCR repertoire whereas CD8 T cells specific for a subdominant (EBNA4 399-408) epitope expressed a narrow range of TCRs [Campos-Lima et al., 1997]. However, substantial variation of TCR repertoire usages by antigen-specific CD8 T cells was observed in other virus disease models [Koning et al., 2013, Utz et al., 1996, Argaet et al., 1994]. For example, in a murine influenza virus model, the TRB repertoire of CD8 T cells specific for a subdominant epitope PB1-F2$_{62-70}$ showed relatively higher diversity than that of CD8 T cells specific for a dominant epitope NP$_{366-374}$. Moreover, there was no obvious difference in the functional avidity of these CD8 T cell populations [La Gruta et al., 2008].

For viruses with a rapid rate of evolution, such as HIV, several studies have presented evidence that diverse TCR repertoires of responding CD8 T cells provide enhanced immune protection against epitope variant escape [Price et al., 2004, Meyer-Olson et al., 2004, Charini et al., 2001]. However, other studies have reported that the affinity of TCRs for the pMHC I determine the likelihood of mutational escape from immune protection [Kloverpris et al., 2015, Ladell et al., 2013, Varela-Rohena et al., 2008]. An affinity-enhanced TCR observed in CD8 T cells of HLA-A2 individuals responding to an immunodominant epitope SL9 derived from HIV Gag antigen were found to recognise all common variants of the epitope, whereas the wild-type TCR provided protection to some but not all variants [Varela-Rohena et al., 2008].

1.2.5.2 Sharing of antigen-specific TCRs by multiple individuals

T cell responses to an epitope usually include T cells expressing different TCR chains involving diverse V, D and J gene rearrangements [Nikolich-Zugich et al., 2004]. In some T cell responses, almost all TCR chains are unique to individual animals/humans. These TCRs are referred to as private TCRs [Kedzierska et al., 2006, Kim et al., 2005, Cibotti et al., 1994]. However, in other responses, the responding cells contain TCRs that are apparently identical in multiple individuals and in some cases dominate the response to the respective antigenic epitope. These are referred to as public TCRs [Venturi et al., 2008a, Dong et al., 2010, Venturi et al., 2008b, Venturi et al., 2006, Kedzierska et al., 2004]. Public TCRs have been detected in CD8 T cell responses specific for epitopes derived from HIV, SIV (Simian immunodeficiency virus) and influenza virus [Gillespie et al., 2006, Price et al., 2004, Kedzierska et al., 2004] and can serve as useful markers for following the antigen-specific CD8 T cell responses. The reasons for induction of public T-cell responses against specific antigens are poorly understood, but recently have been proposed to involve recombinatorial biases and convergent recombination, a process by which multiple recombination events converge to produce the same or similar TCRβ nucleotide sequences that encode the same TCRβ amino acid sequence [Li et al., 2012, Quigley et al., 2010, Venturi et al., 2006]. Public TCRs can be detected through analysis of the rearrangements of epitope-specific TCR α and β chains in

multiple individuals. The immune defence potential of public TCRs is still controversial. For example, conserved TRB clonotypes used by CD8 T cells specific for a Mamu-A*01 presented immunodominant epitope TL8 derived from SIV Tat antigen were found to facilitate epitope variant escape from TCR recognition as a consequence of residue substitution at potential TCR contact sites [Price et al., 2004]. Conversely, protective activity of a public TCR against naturally occurring variants of a Gag-derived CM9 epitope was reported for CD8 T cell responses against SIV [Price et al., 2004]. In a recent study, public TCRs were frequently found in CD8 T cells specific for the most dominant epitopes identified in HIV, whereas CD8 T cell responses to lower ranking epitopes included no or fewer public TCRs [Kloverpris et al., 2015].

1.2.5.3 Correlation between naïve and immune repertoire of antigen-specific CD8 T cells

The development of methods for deep sequencing of TCR repertoires and generation of transgenic mice expressing epitope-specific TCRs, have facilitated studies to investigate the differentiation of naïve T cells following exposure to antigen and the influence of the naïve TCR repertoire on the composition of the responding antigen-specific CD8 T cells. Early studies of activation and differentiation of naïve T cells in TCR-transgenic mouse models revealed recruitment of a broad repertoire of TCR clonotypes into antigen-specific T cell response, with limited skewing of TCR diversity compared to the naïve repertoire [Zehn et al., 2009, Malherbe et al., 2004]. However, selective expansion of T cell clones with high-affinity TCRs for pMHC binding was observed, suggesting that the strength of the TCR/pMHC interaction influenced the clonal composition of responding T cells [Corse et al., 2011, Zehn et al., 2009]. Recently, use of magnetic beads based enrichment of antigen-specific CD8 T cells from conventional mice following staining with pMHC I tetramers has enabled the purification of naïve antigen-specific CD8 T cells for analysis of their TCR profiles [Obar et al., 2008, Moon et al., 2007]. Statistical analysis of epitope-specific TRB repertoires for naïve and immune pMHC I tetramer-positive CD8 T cells indicated that the TCR repertoire of the responding CD8 T cells reflected that in the naïve repertoire, although clear

evidence was observed for preferential expansion of particular clonotypes within the immune CD8 T cell pool [La Gruta et al., 2010]. These results indicate that, at least in some systems, the composition of the naïve epitope-specific TCR repertoire is predictive of the clonal composition of responding T cell populations, but that within this repertoire there is expansion of dominant clonotypes expressing TCRs with high affinity for pMHC I [La Gruta and Thomas, 2013]. It is possible that the extent to which these clonotypes dominate the response depends on the biology of the pathogen inducing the response.

1.3 Immunodominance

Immunodominance is the phenomenon whereby the CD8 T cell response of an individual host to a given pathogen is highly focused on a few antigenic peptides, despite the expression of several MHC I molecules capable of presenting a large number of peptides derived from the pathogen [Yewdell and Bennink, 1999]. This phenomenon has been reported as a common feature of CD8 T cell responses against many intracellular pathogens. Viruses are the most widely used models for studying the biological basis of immunodominance. Activated CD8 T cells proliferate and differentiate to express functional activities that eliminate infected cells or induce intracellular death of the pathogen. The active response is followed by a contraction phase, with substantial apoptosis of the reactive cells and return of surviving T cells to a resting state, resulting in survival of only 5-10% of the antigen-stimulated CD8 T cells as memory cells. The memory CD8 T cells survive in recirculating T cell pool for prolonged time periods ranging from weeks to months in mice or years in humans [Kaech et al., 2002]. Immunodominant responses have been proposed to be advantageous, by allowing the immune system to accommodate a relatively narrow range of memory cell clonotypes specific for a wide range of pathogens encountered throughout life [Vezys et al., 2009].

A well-documented example for immunodominance is the CD8 T cell responses against HIV. Individual CD8 T cell responses have been reported to focus on one or few epitopes [Day et al., 2001, Goulder et al., 1997], and when the immune response selects mutated virus that escape the immune response, a new CD8 T cell response is

generated to a pre-existing epitope that had not previously induced a response [Im et al., 2011, McMichael and Phillips, 1997, Nowak et al., 1995]. The change of immunodominance illustrates an important biological feature of such CD8 T cell responses, namely their contribution to strain specificity.

1.3.1 Epitope dominance hierarchy

Epitope dominance hierarchy refers to the relative magnitude of CD8 T cell responses to different epitopes derived from the same pathogen. Such dominance hierarchies are profound in CD8 T cell responses to many pathogens, such that the detectable response may be focused entirely on 2 or 3 of the epitopes (referred to as immunodominant epitopes), among a larger number of epitopes to which the host is capable of responding. These additional epitopes that generate weak or undetectable responses are referred to as subdominant epitopes [Yewdell and Bennink, 1999].

The dominance hierarchy can have implications for immune protection of the resultant CD8 T cell response. In an influenza virus infected B6 (H-2^b) mouse model, CD8 T cells preferentially respond to one dominant epitope $NP_{366-374}$ derived from the nucleoprotein [Flynn et al., 1999]. The $NP_{366-374}$ epitope-specific CD8 T cells displayed cytotoxic activity against influenza virus-infected cells and were shown to mediate immune protection [O'Neill et al., 2000, Christensen et al., 2000, Allan et al., 1990]. Moreover, later work showed that CD8 T cell responses to a subdominant epitope in the influenza virus (i.e. $PB1_{703}$ epitope derived from the polymerase B subunit) were unable to compensate the immune protection of this dominant epitope [Webby et al., 2003]. Despite the evidence of superior protective activity of dominant compared to subdominant epitopes in some systems, protection mediated by CD8 T cell responses to subdominant epitopes has been reported for some virus diseases, including HIV and LCMV [Im et al., 2011, Frahm et al., 2006, van der Most et al., 2003].

The degree to which dominance hierarchies are maintained in animals or humans expressing the relevant MHC I restriction element varies. Immunodominance tends to be consistent in MHC I-homozygous inbred strains of mice, but can differ in MHC I-heterozygous F1 mice. In some disease models, dominance of particular epitopes

identified in homozygous mice was retained in F1 mice [Kotturi et al., 2008, Liu et al., 2004], whereas inconsistent epitope dominance in different F1 progeny was observed in other disease models [Flesch et al., 2010, Belz et al., 2000, Rutigliano et al., 2007]. In humans expressing the MHC I molecule HLA-A2 (A*0201 allele), CD8 T cell responses to influenza virus are consistently dominated by CD8 T cells specific for the matrix protein epitope MP_{58-66}, regardless of genetic background [Stewart-Jones et al., 2003, Gotch et al., 1987]. However, studies of human CD8 T cell responses to HIV demonstrated that the responses to epitopes, which were dominant in some individuals expressing the restricting MHC I allele, were not dominant in other individuals expressing the same MHC I allele [Betts et al., 2000]. A number of factors shown to influence this variation in epitope dominance are discussed further below.

1.3.2 Factors influencing epitope dominance

Extensive research has indicated that epitope dominance is influenced by multiple interdependent factors involved in almost every step of the antigen-specific CD8 T cell response [Chen et al., 2000, Yewdell and Bennink, 1999]. In general, these factors can be grouped into two broad categories: (1) factors influencing MHC I restricted epitope generation and cell surface expression and (2) factors affecting antigen-specific CD8 T cell activation, proliferation and establishment of memory [Yewdell, 2006, Akram and Inman, 2012]. The relative contribution of each factor appears to vary depending on the disease model.

1.3.2.1 Factors that influence epitope abundance

Studies of CD8 T cell responses to various intracellular pathogens have demonstrated that epitope abundance can influence the magnitude of CD8 T cell responses [Luciani et al., 2013, La Gruta et al., 2006b, Tenzer et al., 2009]. In some systems, this may relate to the levels of expression of the proteins from which the epitopes are generated. Dominant CD8 T cell responses to the intracellular parasite *Trypanosoma cruzi* were shown to be specific for identical epitopes present in several closely related proteins encoded by a multi-gene family [Martin et al., 2006]. The dominance was proposed to be due to the relatively high abundance of these

epitopes because of their presence in multiple proteins. Alternatively, some epitopes may be generated more efficiently than others by proteasome degradation. The amino acid sequences flanking epitopes within a protein have been shown to influence the epitope processing [Tenzer et al., 2009, Le Gall et al., 2007]. Another important factor in determining dominance in some pathogens is the stage of expression of proteins during infection. This is well illustrated by herpesviruses in which the proteins are expressed in an orderly manner – immediate early, early and late, and immunodominant CD8 T cell responses to the immediate early antigens have been reported [Abbott et al., 2013, St Leger et al., 2011, Reddehase and Koszinowski, 1984]. Down-regulation of MHC I expression later in infection was illustrated to partly contribute to this phenomenon. During antigen processing, the influence of TAP on epitope abundance was reported in an HIV model, in which the quantities of several defined epitope precursors were found differ substantially between the cytosol and ER, suggesting preferential transport of certain peptides by TAP [Tenzer et al., 2009].

1.3.2.2 Influence of CD8 T cell abundance and avidity

Studies using several virus disease models have provided evidence that the frequency of naïve antigen-specific CD8 T cells influences the immunodominance hierarchy of antigenic epitopes [Tan et al., 2011, Schmidt et al., 2011, Kotturi et al., 2008]. However, other studies contradicted these findings by demonstrating that the ability of the epitope-specific naïve T cells to proliferate and undergo differentiation following antigen recognition, rather than their frequency, determines the hierarchy of epitope-specific CD8 T cell responses [Cukalac et al., 2014b, Jenkins and Moon, 2012, La Gruta et al., 2010]. For example, in the B6 (H-2^b) mouse model of influenza virus infection, frequencies of naïve CD8 T cell precursors for two subdominant epitopes $D^bPB1\text{-}F2_{62}$ and K^bNS2_{114} were found to be higher than that of two dominant epitopes D^bNP_{366} and D^bPA_{224}. By tracking the recruitment of naïve specific CD8 T cells during primary infection, it was demonstrated that the slow expansion and differentiation of $D^bPB1\text{-}F2_{62}$ epitope-specific naïve CD8 T cells accounted for the subdominant status of this epitope [La Gruta et al., 2010]. A recent study, which analysed T cell affinity (a representative measurement of avidity) of

naïve and immune CD8 T cells for these four epitopes, observed that CD8 T cells specific for the dominant epitopes tend to have higher affinity for pMHC I complexes [Cukalac et al., 2014a]. All these results indicate that both the quality and quantity of naïve CD8 T cells have the potential to influence epitope dominance hierarchy.

1.3.2.3 Influence of genetic background on the TCR repertoire

As discussed above (section 1.2.5), the frequencies and TCR repertoire diversity of epitope-specific CD8 T cells in the naïve T cell pool can greatly influence the magnitude of antigen-specific CD8 T cell responses [Kloverpris et al., 2015, Balamurugan et al., 2010, Turner et al., 2006, Price et al., 2004, Messaoudi et al., 2002]. Since generation of the TCR repertoire involves negative selection of specificities that recognise self, the genetic background of the host can have a strong influence on the composition of the repertoire. Gene products that are highly polymorphic, such as MHC I proteins, are particularly influential. The presence of other MHC I alleles along with the allele that restricts the CD8 T cell response to a particular epitope has been shown to alter the epitope dominance hierarchy in both humans and mice [Betts et al., 2000, Belz et al., 2000]. A reduction of up to 95% in the CD8 T cell response to an H-2D^b-restricted influenza virus epitope was found in F1 MHC I-heterozygous mice compared to homozygous animals. TCR repertoire analysis of the epitope-specific CD8 T cells derived from these mice revealed the deletion of a predominant clonotype in the TCR repertoire of the F1 mice [Belz et al., 2000]. Such deletions are referred to as a "hole" in the repertoire. MHC I-induced TCR repertoire alteration has also been demonstrated in human CD8 T cell responses to EBV; deletion of T cells expressing a dominant TCR β chain specific for an HLA-B8-restricted EBV epitope (FLRGRAYGL) was observed in HLA-B8/HLA-B*4402 heterozygous individuals. The EBV-specific T cells expressing this TRB clonotype were found to cross-react with the HLA-B*4402 alloantigen [Burrows et al., 1995, Argaet et al., 1994]. These results indicate that TCR clonotype can have profound effect on epitope-specific CD8 T cell responses.

1.4 Aims of the project

Previous research has provided evidence that CD8 T cells mediate immunity against infection with *Theileria parva* [McKeever et al., 1994]. However, the immunity induced by one parasite strain doesn't give complete protection against other strains and this has been demonstrated to be associated with parasite strain specificity of the CD8 T cell responses [Taracha et al., 1995b, Morrison et al., 1987]. There is evidence that such strain specificity is a consequence of the CD8 T cell responses of individual animals being focused on a limited number of immunodominant polymorphic peptide-MHC I determinants. Dominant responses to the Tp2 antigen have been demonstrated in animals homozygous for the A10 MHC I haplotype [Connelley et al., 2011, MacHugh et al., 2009]. Three Tp2 epitopes recognised by A10 animals ($Tp2_{49\text{-}59}$, $Tp2_{50\text{-}59}$ and $Tp2_{98\text{-}106}$) have been defined. This project set out to investigate the dominance of these epitopes in A10 animals and, by sequencing of T cell receptor (TCR) genes, investigate the clonal composition of the responding CD8 T cells, with the eventual aim of isolating pairs of TCR α and β chain cDNAs that can be used to generate cells expressing TCRs of defined epitope specificity.

The specific objectives were to: (i) Determine the dominance hierarchies of the three defined Tp2 epitopes in both A10-homozygous and -heterozygous cattle (Chapter 3). To achieve this, MHC I tetramers incorporating epitope-related peptides were used for rapid quantification of epitope-specific CD8 T cells within responding populations. (ii) To determine the clonal repertoires of epitope-specific CD8 T cell populations by sequencing the expressed TRB genes, in order to examine the potential contribution of CD8 T cell clonal composition to epitope dominance hierarchy (Chapter 4 and 5). (iii) To isolate full-length cDNAs encoding TCR α and β chain pairs from T cell clones of defined epitope specificity and use them to generate cells expressing the functional TCRs. This part of the work (Chapter 6) aimed to provide the basic tools for generating naïve antigen-specific CD8 T cells, which could be used in further studies to investigate the requirements for induction of primary antigen-specific CD8 T cell responses *in vitro*.

Chapter 2: Material and methods

2.1 Experimental animals

Three MHC I A10 homozygous and six A10 heterozygous Holstein animals were immunized with the Muguga isolate of *T. parva* by the infection and treatment protocol as described previously [Radley et al., 1975b]. In brief, cattle were infected with cryopreserved *T. parva* (Muguga) sporozoites and at the same time given a long-acting formulation of oxytetracycline, at a dose of 20mg/ml intramuscularly. The MHC class I haplotypes of these animals were determined by initially screening with a panel of MHC I-specific monoclonal antibodies or class I allele-specific PCRs followed by sequencing of the PCR products and are shown in Table 2-1.

Table 2-1 MHC class I haplotypes of animals used in this project.

Animal	MHC class I haplotype	MHC class I alleles
302186	A10/A10	2*01201, 3*00201
403992	A10/A10	2*01201, 3*00201
403957	A10/A10	2*01201, 3*00201
102121	A10/A12	2*01201, 3*00201, 1*01901, 2*00801
402145	A10/A12	2*01201, 3*00201, 1*01901, 2*00801
102170	A10/N5	2*01201, 3*00201, 3*03601, 3*03701
702162	A10/N5	2*01201, 3*00201, 3*03601, 3*03701
402082	A10/A11	2*01201, 3*00201, 2*01801, 3*01701
702197	A10/A11	2*01201, 3*00201, 2*01801, 3*01701

2.2 Cellular techniques

2.2.1 Isolation of peripheral blood mononuclear cells

A volume of 60ml of blood was obtained by jugular venupuncture and collected into syringes containing 3-4ml of PBS with 0.5mM EDTA solution, which served as an anti-coagulant and diluent. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll-Paque as described before [Goddeeris and Morrison, 1988]. Aliquots of 30ml of the blood/EDTA solution

mixture were carefully overlaid onto 20ml of Ficoll-Paque Plus (GE Healthcare Life Sciences) in 50ml polypropylene tubes and centrifuged at 900×g for 30min at room temperature. PBMC were harvested from the Ficoll-Paque Plus/plasma interface and mixed with an equal volume of PBS containing 2mM EDTA before pelleting by centrifugation at 450×g for 10min at room temperature. The pellets were washed three times in PBS containing 2mM EDTA, counted and resuspended in a suitable volume of standard cell culture medium (SCM - RPMI 1640 containing 10% foetal bovine serum (FBS), 5×10^{-4} M 2-mercaptoethanol (2-ME), 100U/ml penicillin, 100µg/ml streptomycin and 0.29mg/ml L-glutamine) for subsequent use. If necessary, PBMC containing residual red blood cells (RBCs) were subjected to osmotic lysis prior to the second wash by re-suspending the pellet in 5ml of RBC lysis buffer (17.5mM TRIS, pH7.4 with 144mM ammonium chloride) pre-heated to 37°C, and then incubating at 37°C for 2-3min, followed by the third wash as described above.

2.2.2 CD8 T cell purification from PBMC

Magnetic-activated cell sorting (MACS) was conducted according to the manufacturer's instructions (Miltenyi Biotec). Briefly, 1×10^8 PBMC were pelleted by centrifugation at 400×g for 5min at 4°C and re-suspended in 5ml of IL-A105 hybridoma culture supernatant (antibody specific for CD8 T cells, titrated before use) at a pre-determined dilution. After incubation for 10min at room temperature, cells were washed twice with PBS and centrifuged at 400×g for 5min at 4°C. After the second wash, fluid above the cell pellet was removed completely. Pelleted cells were re-suspended directly in a suspension of micro-beads coated with anti-mouse IgG (Miltenyi Biotec, 100µl per 1×10^8 cells) and incubated for 10min at room temperature. Cells were washed twice with PBS (10mls/10^8 cells) and re-suspended in 0.5ml MACS buffer (PBS with 0.5% bovine serum albumin (BSA) and 2mM EDTA) for MACS purification. An MS column (reservoir volume 3.5ml) was placed on the separator magnet (Miltenyi Biotec) and washed with 0.5ml MACS buffer, before adding the prepared suspension of beads and cells to the reservoir at the top of the column. Cells bound to the column were washed three times with 0.5ml of MACS buffer to remove unbound cells and 2ml SCM added to the top of the column before it was removed from the magnet and placed to the top of a new 15ml tube

containing 5ml SCM. CD8 T cells were collected by plunging the SCM through the column. After centrifugation at 400×g for 5min, pelleted cells were re-suspended in appropriate SCM for subsequent use. The purity of sorted CD8 T cells was determined by flow cytometry cell analysis as described in section 2.2.8.

2.2.3 Depletion of CD4 and γδ T cells from cultures

Cells were re-suspended in monoclonal antibodies IL-A12 (specific for bovine CD4) and GB21A (specific for bovine γδ T cells) at predetermined dilutions with a final density of 1×10^7/ml in a 15-ml Falcon tube and incubated for 30min at 4°C on a rotating mixer at 20 rpm and washed once with SCM. 4×10^7 Dynal beads coated with anti-mouse Ig antibody (Life Technologies) were added per 1×10^7 cells in 1ml. Cell/bead mixture was re-suspended in SCM at 5×10^6 cells/ml in a 15-ml Falcon tube and incubated for 30min at 4°C on a rotating mixer at 20 rpm. The volume was expanded to 10ml with SCM and the tube placed in the magnet for 2min to immobilise the bead-bound labelled cells. Unlabelled cells were collected and re-suspended in an appropriate volume of SCM.

2.2.4 *T. parva*-infected cell lines

Cell lines infected with *T. parva* (Muguga) (TpM) from the 6 animals heterozygous for the MHC I A10 haplotype listed in Table 2-1) and from an additional A10-homozygous animal (592) and an A14-homozygous animal (605) had been generated previously and were available from cryopreserved stocks. *T. parva* (Muguga) transformed cell lines (TpM) were established for 3 further A10-homozygous animals (302186, 403957 and 403992, Table 2-1) by *in vitro* infection of PBMC with sporozoites. 2×10^7 PBMC were pelleted by centrifugation at 200×g for 5min, re-suspended in 1ml of a 1:3 dilution of *T. parva* (Muguga) sporozoites (stabilate 80) in SCM and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 1.5 h with gentle mixing every 30minutes. The cells were then washed once in 10ml SCM, re-suspended in 10ml of SCM, distributed into a 24 well plate (2ml per well) and incubated in a humidified atmosphere of 5% CO_2 at 37°C. The cells were monitored microscopically for growth of transformed lymphoid cells. Once established, TpM cell lines were expanded into culture flasks and kept in a

humidified atmosphere of 5% CO_2 at 37°C. Established cultures were maintained by removal of 50-75% of the volume of the cultured cell suspensions every 2-3 days and replacing it with fresh SCM. The phenotypes of the TpM cell lines were determined by flow cytometry using a panel of monoclonal antibodies specific for CD3, CD4, CD8, γδ T cells and IgM as described below (section 2.2.8 and Table 2-2).

2.2.5 *In vitro* generation of *T. parva*-specific CD8 T-cell enriched cell lines

The protocol for generation of *T. parva*-specific CD8+ T cell enriched cell lines was adapted from that described previously [Goddeeris and Morrison, 1988]. The protocol involved 3 stimulations of PBMC with autologous irradiated infected cells at 7 day intervals.

First stimulation: PBMC were isolated and counted as described above. Autologous TpM cells (stimulators) were harvested, counted and exposed to 60Gy of gamma irradiation from a ^{137}Caesium source. Into each well of a 24-well plate were placed 2ml of SCM containing 2×10^6 PBMC/ml and 1×10^5/ml irradiated autologous stimulators. Plates were incubated for 7 days in a humidified atmosphere of 5% CO_2 at 37°C.

Second stimulation: Cells from the first stimulation were harvested, counted and reseeded into 24-well plates in 2ml of SCM at a density of 2×10^6/ml together with irradiated autologous stimulators at 1×10^5/ml. Plates were incubated for 7 days in a humidified atmosphere of 5% CO_2 at 37°C.

Third stimulation: Cells from the second stimulation were harvested, counted and the CD4+ and γδ T cell populations depleted by antibodies and anti-mouse Ig coated beads as described in section 2.2.3. The remaining cells were washed, resuspended in SCM and re-seeded into 24-well plates in 2ml of SCM at a density of 1×10^5/ml together with irradiated stimulators at a density of 5×10^5/ml and 100U/ml recombinant human IL2 (rIL2, Novartis international AG, USA), and incubated for 7 days in a humidified atmosphere of 5% CO_2 at 37°C.

Some experiments involved *in vitro* stimulation of CD8 T cells purified from PBMC by MACS separation as described above. The CD8 T cells were seeded into 24-well plates in 2ml of SCM at a density of 1×10^5/ml together with autologous stimulators at a density of 5×10^5/ml and 100U/ml rIL2, and incubated in a humidified atmosphere of 5% CO_2 at 37°C.

The generated CD8 T cell lines, subjected to three *in vitro* stimulations, were cryopreserved (section 2.2.7), while some lines were maintained in culture by further *in vitro* stimulation at a density of 1×10^5/ml together with irradiated stimulators at a density of 5×10^5/ml and 100U/ml rIL2.

2.2.6 Cloning of *T. parva*-specific CD8 T cells

Cells obtained following the third stimulation and/or from the purified CD8 T cells after a single stimulation with infected cells were harvested and assessed for CD8 T cell purity by flow cytometry (see section 2.2.8). If there was significant contamination (>2%) with γδ T cells and/or CD4 T cells, the cell lines were subject to another round of depletion by staining with antibodies specific for CD4 and γδ TCR and removal with anti-mouse Ig-coated Dynal beads as described above (see section 2.2.3), until >98% of the population were CD8 T cells. The CD8 T cells were cloned by limiting dilution. They were serially diluted in SCM to give cell densities of 40, 20, 10, 5, 2.5 cells/ml. Aliquots of 100µl of each of these suspensions were distributed into the wells of a 96-well round-bottom plate, to give final densities ranging from 4 to 0.25 cells/well. To each well, was added a 100µl aliquot of SCM containing 5×10^4/ml irradiated autologous stimulators, 2×10^5/ml irradiated autologous filler cells (PBMC) and 200U/ml rIL2. Plates were incubated for 2 weeks in a humidified atmosphere of 5% CO_2 at 37°C, after which the wells were screened microscopically and wells displaying obvious cell growth were selected for further expansion of the cells. Only wells from plates showing growth in <30% of wells were selected, as they have greater than 83% possibility of being clonal [Goddeeris and Morrison, 1988].

For *in vitro* expansion of clones, selected clones were transferred to individual wells of 48-well plates containing 2.5×10^5 irradiated autologous stimulators and

100U/ml rIL2 in a total volume of 1ml SCM. Plates were incubated in a humidified atmosphere of 5% CO_2 at 37°C and clones were re-stimulated in the same way every two weeks as required.

2.2.7 Cryopreservation of cells

Parasitized cell lines and T cell lines to be cryopreserved were pelleted, re-suspended at 2×10^6 cells/ml in 90% heat-inactivated foetal bovine serum (FBS) containing 10% dimethylsulphoxide (DMSO) and 1ml aliquots transferred into 2ml polypropylene cryovials. These vials were placed into an isopropanol jacketed container and slowly frozen at -70°C before being transferred to liquid nitrogen storage. CD8 T cell lines were routinely cryopreserved two days after stimulation with irradiated parasitized cells. For further use of the cryopreserved cells, they were rapidly thawed by incubation in a water bath at 37°C, washed in SCM and re-suspended in the original (pre-freeze) volume of SCM for further culture.

2.2.8 Flow cytometry analysis

The monoclonal antibodies (mAb) used for primary labelling of cells for phenotypic analysis are listed in Table 2-2. Hybridoma culture supernatants producing the mAb were used at a dilution of 1:5 in FACS medium (RPMI 1640 medium with 2% foetal bovine serum and 0.2% Sodium Azide), while those available in the form of ascitic fluid were used diluted 1:1000 in FACS medium.

Aliquots of 50µl of cell suspensions at 2×10^7 cells/ml were distributed in wells of 96-well round-bottom well plates and 50µl of the required primary mAb added. Plates were incubated at 4°C for 30min, washed three times in FACS medium and then re-suspended in 50µl of fluorescein-isothiocyanate (FITC)-labelled goat polyvalent anti-mouse immunoglobulin G, A and M antibody (Sigma-Aldrich, Poole, Dorset, UK). Following incubation at 4°C for 30min, the cells were washed three times in FACS medium and then re-suspended in 200µl FACS medium for analysis on a BD FACSCalibur cell analyser (BD Biosciences). Negative controls were incubated with FACS medium instead of primary labelling antibody.

Table 2-2 Monoclonal antibodies used for primary labelling in FACS analysis

Antibody	Isotype	Specificity	Cell distribution
MM1A	IgG1	CD3	T-cells
IL-A12	IgG2a	CD4	$CD4^+$ T cells
IL-A105	IgG2a	CD8	$CD8^+$ T cells, NK cells
GB21A	IgG2b	γδ TCR	All γδ T cells
IL-A30	IgG1	IgM	B cells
Gr13	IgG1	Nkp46	NK cells
QBEND-10	IgG1	Human CD34	Lentivirus-transduced cells

2.2.9 Tetramer staining and cell sorting

In vitro generated cell lines enriched for CD8 T cells were harvested and re-suspended in SCM at a density of 1×10^7cells/ml. Aliquots of 100µl cell suspension were distributed in wells of 96-well round-bottom well plates, centrifuged briefly and re-suspended in 50µl of 20nM tetramers. Phycoerythrin (PE) labelled MHC I tetramers that incorporate three defined epitopes derived from the Tp2 antigen ($Tp2_{98\text{-}106}$, $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$) associated with the BoLA 2*01201 heavy chain were used. After incubation at 4°C for 30min in the dark, cells were washed twice with FACS medium and re-suspended in 200µl FACS medium for analysis using a BD FACSCalibur. Negative controls were incubated with FACS medium instead of tetramers.

For cell sorting of tetramer-specific CD8 T cells, preliminary tetramer staining was undertaken to quantify the Tet^+CD8^+ T cells to enable calculation of the total number of cells needed for staining with the respective tetramers for cell sorting. Cells were suspended in SCM medium at a density of 1×10^7cells/ml and stained with tetramers as described above. Keep cells sterile if subsequent culture is needed. Cell sorting was performed on BD FACSAria IIIu.

2.2.10 Culture of 293T cells

HEK 293T adherent cells were cultured in DMEM medium with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin. Cells were subcultured when confluent,

every 2-3 days. The 293T cells were harvested from T75 flasks with a volume of 15ml medium per flask by first gently flushing them with PBS and then adding 3-5ml of 0.25% Trypsin-EDTA dissociation reagent (Life Technologies) and incubating in a humidified atmosphere of 5% CO_2 at 37°C for 1-2minutes. 10ml of DMEM medium was added immediately after incubation to wash off the cells. The cell suspension was harvested, pelleted by centrifugation and the cells counted and used as required.

2.2.11 Culture of Jurkat cells

The Jurkat cell line JRT3-T3.5, a human TCR-negative CD8 T cell line transfected with the luciferase reporter gene under the transcriptional control of NFAT (nuclear factor of activated T cells)-responsive elements and the Neomycin-resistance gene, was generously provided by Dr. John Bridgeman, Cardiff University. The cell line was cultured in RPMI-1640 medium supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin and 0.29mg/ml L-glutamine. Selective antibiotic G418 (for the Neomycin-resistance gene) with a final concentration of 50µg/ml was added into the culture to maintain the cell line.

2.2.12 Production of lentivirus in 293T cells

For transfection, 1.5-2×10^7 293T cells were seeded into a T75 culture flask the day before transfection. Freshly prepared culture medium at pH7.1 (25mM HEPES Buffer in serum free DMEM medium, adjust pH to 7.1 and filter with 0.2µm filter), and at pH7.9 (25mM HEPES Buffer in DMEM medium+10% FBS, adjust pH to 7.9 and filter with 0.2µm filter) were used for the transfection procedure. 1M $CaCl_2$ was prepared by dissolving 7.35g hydrated $CaCl_2$ in 50ml water, filtered through a 0.2µm filter and stored at -20°C in single use aliquots. For lentivirus production, 30µg pSxW lentiviral-TCR transfer vector, 30µg pCMVΔ8.91 and 15µg pMD2.G (vectors were given by Dr. John Bridgeman, volume for each vector was calculated according to the concentration of purified plasmid in 10mM Tris·Cl, pH 8.5) were added to a 15ml Falcon tube in medium at pH 7.1 to a volume of 2.85ml; 150µl of $CaCl_2$ was then added. The tube was vortexed and left for 10-30min at room temperature. Medium was removed from flasks of cultured 293T cells before gently adding

12.0ml of pH 7.9 medium. The transfection mix was briefly vortexed and added to the 293T cells dropwise. Cells were then cultured in a humidified atmosphere of 5% CO_2 at 37°C for 24 hours. Replace the media with 20ml RPMI-1640 medium supplemented with 10% FBS, 4mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin and cells were cultured for another 24 hours. Supernatant was then collected and filtered through a 0.45µm filter and stored at 4°C. Cells were continually cultured in 20ml of fresh RPMI-1640 medium supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin and 4mM L-glutamine. The second collection of supernatant was made after 24 hours and filtered through a 0.45µm filter. The two collections were centrifuged at 24000 xg for 2 hours at 4°C in ultra-clear ultracentrifuge tubes (Beckman Coulter). Following centrifugation, the supernatant was decanted and the pellet was re-suspended in a 10-fold smaller volume than the original supernatant volume. Aliquots were snap frozen on dry-ice and stored at -80°C.

2.2.13 Lentiviral transduction of Jurkat cells

The day before transduction, 1ml of 1×10^6 JRT3-T3.5 cells were seeded into each well of a 24-well plate. After overnight culture at 37°C, the supernatant was replaced with 1ml of viral stock (virus titre was determined according to the description in section 6.2.3) for transduction. Polybrene (Hexadimethrine bromide, Sigma) was added to a final concentration of 6µg/ml. A stock solution of Polybrene, prepared by dissolving in H_2O at 6mg/ml, was filtered through 0.2µm filter and stored in aliquots at -20°C.

Transcription of the TCR by transduced JRT3-T3.5 cells was determined 72h post-transduction, by staining the cells by indirect immunofluorescence for surface expression of the transcribed marker gene product human CD34. Transduced and un-transduced Jurkat cells were stained with CD34-specific antibody QBEND-10 (Abcam). FACS analysis was applied as described in section 2.2.8.

2.2.14 Lentiviral transduction of bovine CD8 T cells

Bovine CD8 T cells were purified from PBMC by MACS sorting, as described in section 2.2.2. Purified CD8 T cells were activated by incubating the cells in anti-bovine CD3-coated wells, to which SCM containing anti-bovine CD28 (5µg/ml) and rIL2 (100U/ml) were added, as described previously [Nijhuis et al., 1990, Hogg et al., 2011]. The cells were incubated for 24 hours in a humidified atmosphere of 5% CO_2 at 37°C. The time line for pre-stimulation and transduction of bovine CD8 T cells was as follows:

Day 0 – Plate coating: Anti-bovine CD3 antibody (MM1A) diluted in PBS at a final concentration of 1µg/ml was added to a 96-well flat bottom untreated microtitre plate (Corning, #3370). The plate was incubated at 4°C overnight to allow antibody binding.

Day 1 – Pre-stimulation of bovine CD8 T cells: The coating antibody was removed from each well of the coated plate and 5×10^4 purified CD8 T cells added along with anti-bovine CD28 antibody at 5µg/ml (AbD Serotec, clone CC220, Low Endotoxin) and rIL2 at 100U/ml (final concentrations) in 200ul of SCM. Cells were incubated at 37°C in 5% CO_2 for 24 hours.

Day 2 – Lentiviral transduction of bovine CD8 T cells: The supernatant was carefully removed from the wells and virus added directly to the cells at a multiplicity of infection (MOI) ranging from 1~10. For example, 250µl of virus with a titer 2×10^6 TU/ml added to 5×10^4 cells resulted in an MOI=10. Polybrene and rIL2 were added to give final concentrations of 6µg/ml and 100U/ml respectively. Cells were cultured at 37°C in 5% CO_2 for 48-72 hours before monitoring for transgene expression.

2.3 Molecular techniques

2.3.1 Template-switch anchored reverse transcription-polymerase chain reaction (RT-PCR) for TRB repertoire analysis

The method was adopted from previously published work about TCR repertoire analysis [Quigley et al., 2011, Douek et al., 2002]. As shown in Figure 2.1, the

involvement of a switching mechanism at the 5'-terminus of the RNA transcript (SMART) facilitates the enrichment of full-length TCR transcripts. The anchor sequence added during reverse transcription allows unbiased amplification of TCR rearrangements using the rapid amplification of cDNA ends (RACE) PCR. The whole procedure includes the following steps: (1) purification of antigen-specific T cell populations for TCR gene analysis; this step was done according to section 2.2.9; (2) mRNA isolation; (3) cDNA synthesis; (4) RACE-PCR amplification of rearranged TCR products; (5) gel extraction and purification of amplicons; (6) amplicon ligation into a TA cloning vector; (7) vector transformation into competent *E. coli*; and (8) colony PCR and sequencing.

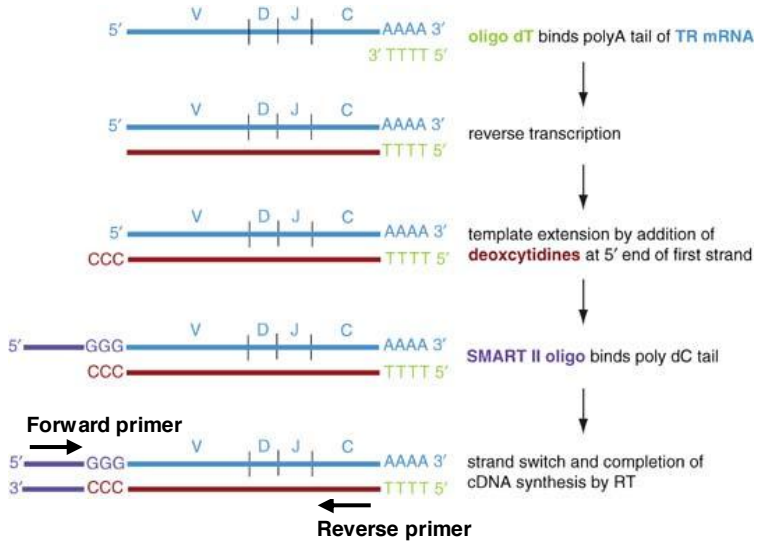


Figure 2.1 Schematic instruction of the 5'-SMART-RACE PCR. The reverse transcription and template switching properties of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase enable the incorporation of an anchor sequence to the 5'-end of full length mRNA during cDNA synthesis. When reverse transcription reaches to the 5'-end of mRNA, the reverse transcriptase first add a short deoxcytidine (dC) sequence to the 3'-end of cDNA. A modified oligonucleotide sequence with G ribonucleotides hybridizes to dC sequence and then reverse transcription switch template to complete cDNA synthesis. With designed forward and reverse primers according to the anchor sequence and constant region of TCR, rearrangements are amplified.

2.3.1.1 mRNA isolation from cells

Purification of mRNA was carried out using the Oligotex Direct mRNAmini Kit (Qiagen) according to the manufacturer's instructions. Briefly, 1×10^6 cultured or

purified cells that were stored in RNAlater were partially thawed and centrifuged immediately at 11,000×g, 4 ℃ for 10min to remove supernatant. The pelleted white precipitate was lysed in buffer OL1 (1ml contains 30µl of β-ME, 600µl added to 1×10^6 cells). The sample was homogenized using a QIAshredder spin column. Binding buffer ODB was added to the lysate, followed by addition of 20µl Oligotex suspension (containing polystyrene–latex beads coated with dT30 oligonucleotides) and the mixture incubated at room temperature for 10min to hybridize the oligo dT30 on the beads with the $polyA^+$ RNA. After centrifugation at 14,000×g for 5min, the pelleted beads were resuspended in 350µl washing buffer OW1 and the sample transferred on to a small spin column. The beads were collected from the column and washed twice with 350µl buffer OW2. mRNA was eluted from the beads by addition of 20µl hot (70°C) buffer OEB and used immediately for cDNA synthesis or stored at −80°C for future use.

2.3.1.2 Reverse transcription – complementary DNA (cDNA) synthesis

The SMARTer™ RACE cDNA Amplification Kit (Clontech) was used, which includes a modified anchor SMARTer II A Oligonucleotide 5'-AAGCAGTGGTA TCAACGCAGAGTACGCGGG-3' with three G residues at the end and a 5'- RACE CDS Primer A (5' CDS) 5'-$(T)_{25}$VN-3' (V=A,C,G; N=A, C, G, T). cDNA synthesis was performed according to the manufacturer's instructions. One microliter of 5'CDS primer and 2.75µl of mRNA were added to a 0.2ml PCR tube and incubated at 72°C for 3min and then 42°C for 2min. Following incubation, 1µl of SMARTer II A oligo and a Master Mix composed of 2µl 5×First-Strand Buffer, 1µl DTT, 1µl dNTP Mix, 0.25µl RNase Inhibitor and 1µl SMARTScribe Reverse Transcriptase were added in a total volume of 10µl. The mixture was incubated at 42 °C for 90min and 70°C for 10min in a hot-lid thermal cycler. The product was then diluted by addition of 15µl Tricine-EDTA buffer and used immediately for RACE PCR amplification or stored at -20°C for future use.

2.3.1.3 RACE PCR amplification of rearranged TRB products

For amplification of rearranged bovine TRB products, a reverse primer located near the 5' end of the constant region of bovine TRB (TRBC) was synthesized as 5'-GGAGATCTCTGCTTCCGAGGGTTC-3'. The forward primer, provided in the

SMARTer™ RACE cDNA Amplification Kit and named as 10× Universal Primer A Mix (UPM), consisted of a mixture of Long (0.4 μM) 5'- CTAATACGACTCACTATAGGGCA AGCAGTGGTATCAACGCAGAGT -3' and Short (2 μM) 5'- CTAATACGACTC ACTATAGGGC-3' versions of the primer. The Advantage® 2 PCR Kit (Clontech) was used for RACE PCR. The reaction was prepared as follows: 10×Advantage 2 PCR Buffer 5μl, 10×UPM 5μl, TRBC (25 μM) 1μl, dNTP 1μl, cDNA 7-13μl, AdvanTaq2 1μl and PCR-Grade H_2O made up to a total of 50μl. The reaction was run in a thymocycler under the following conditions: 1 cycle of 95°C 30sec; 5 cycles of 95°C 5sec and 72°C 2min; 5 cycles of 95°C 5sec, 70°C 10sec and 72°C 2min; 30 cycles of 95°C 5sec, 66°C 10sec and 72°C 2min. The entire product was loaded, along with a 100bp DNA ladder, on a 1.5% agarose gel made with 1×TAE Buffer containing GelRed DNA stain to allow visualization under UV light. The expected product should be within 500-700bp range. The band of interest (expected size range 500-700bp) was excised from the gel using a scalpel and transfered to a clean 1.5ml microcentrifuge tube.

2.3.1.4 Purification of amplicons

NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel) were used for purification of TRB amplicons from agarose gels, according to the manufacturer's instructions. Two volumes of NT buffer were added to one volume of gel and incubated at 50°C for 5-10min to dissolve the gel. The resultant solution was loaded onto the binding column and centrifuged at 11,000×g for 1min to bind DNA to the column membrane. The membrane was washed twice with NT3 buffer (with added ethanol) and DNA eluted with 15-30μl of DNase-free water.

2.3.1.5 Ligation of PCR amplicons into pGEM-T Easy vector

The pGEM-T Easy Vector System I Kit (Promega) was used for ligation. A reaction consisting of 5μl gel-extracted DNA product, 1μl pGEM-T Easy vector, 1.5μl T4 Ligase and 7.5μl 2× Ligation buffer was set up in a 0.5ml microcentrifuge tube. A negative control tube used 5μl H_2O in place of DNA and a positive control included a known positive insert. The reaction was incubated at 4°C for 12-18 h and the prduct used for transformation.

2.3.1.6 Transformation of ligation product into *E.coli* competent cells

The *E. coli* JM109 strain was used for transformation. Ampicillin-containing LB agar plates were used to select transformed bacterial colonies and blue/white screening was used to identify colonies with inserts. The *E. coli* cells were slowly thawed on ice. An aliquot of 50µl competent cells was added to 7.5µl of ligation reaction and gently flicked to mix, avoiding pipetting. The sample was incubated on ice for 30min, heat-shocked for 50 sec at 42°C in a water bath, and placed on ice for a further 2min before adding 950µl SOC (Super Optimal broth with Catabolite repression, components are listed in Appendix A) medium. The sample was placed in a thermomixer (200 rpm), incubated at 37°C for 1.5-2 h and the bacteria spread evenly over the surface of LB plates containing ampicillin (100µg/ml), isopropyl β-D-1-thiogalactopyranoside (IPTG, 50µg/ml) and X-galactosidase (X-gal, 50µg/ml). The plates were inverted and incubated at 37°C overnight (16-24 h).

2.3.1.7 Colony PCR and sequencing

Single white colonies were selected to amplify and sequence the inserts. First, restreak plates were prepared by drawing a 96-well grid on LB plates containing ampicillin, IPTG and X-gal as above. Ninety-five white colonies and one dark blue colony were picked and spread gently within each grid square of a restreak plate. The plates were incubated at 37°C overnight (16-24 h) and a colony from each grid square selected for PCR amplification. The picked colonies were each added to 20µl PCR-grade water in the wells of a 96-well PCR plate and incubated at 98°C for 10min. The plate was then centrifuged at 2000×g for 5min to remove bacterial debris and 2µl supernatant from each well was used as PCR template.

The PCR utilized Master Mix consisting of 10×PCR Buffer 200µl, dNTP 100µl, M13F primer (2.5 µM) 100µl, M13R primer 100µl, BIOTAQ (5units/µl Bioline, London, UK) 10µl and H_2O 1290µl. The Master Mix was distributed into a new 96-well PCR plate, 18µl/well and add 2µl of the colony supernatant added. The PCR used M13F 5'-TTTTCCCAGTCACGAC-3' and M13R 5'-CAGGAAACAGCTATGAC-3'ass the forward and reverse primers respectively. The PCR reaction conditions were as follows: 1 cycle of 95°C for 5min; 35 cycles of 95°C 30sec, 57°C 30sec and 72°C 3min; hold at 4°C.

After amplification, the products were diluted with 20µl sterile H_2O and 5µl of several individual products electrophoresed on an agarose gel to assess the efficiency of amplification. Selection of individual wells was according to a published protocol [Quigley et al., 2011] and shown in Figure 2.2. Samples with the expected band size were selected for Sanger sequencing at Edinburgh Genomics.

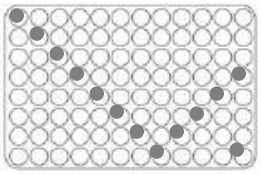


Figure 2.2 Selection of wells for checking colony PCR efficiency. 5µl of diluted PCR product from highlighted wells were used for running on a 1.5% agarose gel. Right bottom corner was PCR product amplified from negative control colony. Expected bands should be visible at approximately 800bp for white colonies and 300bp for dark blue colony.

2.3.2 Analysis of sequence data

An 'in-house' database for bovine TCR genes has been generated by Dr. Timothy Connelley using DNAsis V3.0 software (Miriabio Inc., Alameda, CA). The database includes 86 cDNA transcripts of Vβ genes assigned to 24 subgroups, 17 genomic sequences of Jβ genes assigned to 3 subgroups, 3 genomic sequences of Dβ genes (genome accession number AC_000161 in assembly Bos_taurus_UMD_3.1.1) and 3 genomic sequences of Cβ genes [Connelley et al., 2009]. For bovine TCR α genes, the database includes 306 genomic sequences of Vα genes (GenBank accession numbers JX065635-JX065739) assigned to 36 subgroups, 62 genomic sequences of Jα genes and 1 genomic sequence for Cα gene. TRB transcripts obtained by Sanger sequencing were analysed using DNAsis Max V3.0 software according to the following steps and criteria. In line with previously published work on bovine TCRβ genes [Connelley et al., 2008, Houston et al., 2005], the WHO-IUIS nomenclature system has been used here [Kazatchkine, 1995].

(i). Identification of Vβ genes. For convention, bovine Vβ genes in the database have been given subfamily names according to the human Vβ gene with which they share highest nucleotide similarity [Connelley et al., 2008, Houston and Morrison,

1999]. Sequences obtained in this study were compared with identified bovine Vβ genes in the "in-house" database using the BLASTN algorithm and those sharing <98% nucleotide identity were treated as novel genes and added to the database. Features of the predicted mature Vβ polypeptide, including the presence of conserved amino acid residues at position 23 (cysteine, Cys/C), 41 (tryptophan, Trp/W), 42 (tyrosine, Tyr/Y) and 104 (cysteine, Cys/C), were confirmed. The sequence beyond amino acid 104 and the 3' end of the V gene could have been altered during somatic recombination.

(ii). Identification of Jβ genes. The Jβ gene sequences in the database have been annotated according to the genomic location in the bovine TCRB locus [Connelley et al., 2009]. Using the BLASTN algorithm, sequences from this study were compared with identified bovine Jβ genes in the database. The conserved Phe-Gly-X-Gly (F-G-X-G) motif represents germline Jβ sequence. Sequence beyond the 5' of this motif could have been altered during somatic recombination.

(iii). Presentation of TCRβ chain sequence data: Sequences of bovine TRB transcripts were presented in the standardised format, in which the identified Vβ and Jβ gene segments expressed, the framework sequences flanking the CDR3β and the CDR3β itself are displayed [Chothia et al., 1988].

Bovine TCRα sequences obtained in this study were analysed following the same steps and also presented in the same standardised format.

2.3.3 Prepare samples for high-throughput sequencing (HTS)

2.3.3.1 CD8 T cells purification

PBMC from *T. parva*-immunized or naïve $A10^+$ cattle were prepared (see section 2.2.1) and used for CD8 T cell purification (see section 2.2.2). For high-throughput sequencing, 1×10^7 CD8 T cells were purified from each sample. The purity of sorted CD8 T cells was analyzed by flow cytometry to check for CD4 T cell contamination.

2.3.3.2 mRNA isolation from sorted CD8 T cells

mRNA isolation was carried out according to details described in section 2.3.1.1.

2.3.3.3 Reverse transcription for cDNA synthesis

The Promega® Reverse Transcription System (Promega, UK) was used according to the manufacturer's instructions. Briefly, the reagents listed in Table 2-3 were added to a microcentrifuge tube (0.2µl) and the reaction incubated in a PCR machine as follows: 42°C for 1 hour, then heat at 95°C for 5min followed by incubation at 4°C for 5-10min. The last two stages inactivate the AMV (Avian Myeloblastosis Virus) reverse transcriptase and stop it binding to the cDNA. cDNA was stored at -20°C until use.

Table 2-3 Reaction for Promega Reverse Transcription cDNA synthesis.

Reagent	Volume (µl)
$MgCl_2$, 25mM	4
10×Reverse Transcription Buffer	2
dNTP mixture, 10mM	2
Recombinant RNAsin Ribonuclease Inhibitor	0.5
AMV reverse transcriptase (25 unit/µl)	0.6
Oligo$(dT)_{15}$ primer (0.5µg/µl)	1
mRNA or RNA	1 µg
Nuclease free water to a final volume of 20µl	

2.3.3.4 Preparation of amplicons for sequencing

Primers were designed for amplifying TCR β chain rearrangements as described in chapter 4 section 4.2.3. The Phusion® high-fidelity PCR Kit was used for PCR amplification according to the manufacturer's instructions. The PCR reaction consisted of 5× Phusion HF Buffer 4µl, 10mM dNTP 0.4µl, 10µM Forward Primer 0.4µl, 10µM Reverse Primer 0.4µl, template cDNA 2µl, DMSO 0.6µl, Phusion DNA Polymerase 0.2µl, nuclease-free water to a final volume of 20µl. The mixture was gently mixed and the PCR reaction run in a thermocycler under the following conditions: 98°C 30 sec; 30 cycles of 98°C 5 sec, 62°C 10 sec, 72°C 15 sec; 72°C 5min and store at 4°C. Volumes were scaled up accordingly for 50µl or 100µl

reactions. The optimal PCR conditions had been determined by testing a range of primer concentrations, annealing temperatures and PCR cycles.

2.3.3.5 Purification of PCR product

Agencourt AMPure Beads (Beckman Coulter, High Wycombe, UK) were used to purify PCR amplicons. Briefly, beads equilibrated to room temperature were added to the PCR reaction. Preliminary testing of several volume ratios of beads to PCR reaction demonstrated that use of 1µl of beads for every 1µl of reaction was optimal. Here, 50µl beads were mixed thoroughly with 50µl PCR reaction for 10 times and incubated at room temperature for 15min. The tube was placed on the magnetic stand at room temperature for 2min or until the fluid phase had cleared. The supernatant was removed and the beads were washed two times with 200µl of freshly prepared 75% ethanol. The tube was left to stand for 5min to dry and then removed from the magnetic stand. The dried beads were resuspended in 20µl of 10mM Tris-HCL (pH8.0) buffer, incubated for 2min at room temperature and placed on the magnetic stand until the supernatant was clear. The clear supernatant was transferred to a fresh tube and sent for sequencing or stored at -20°C for future use.

2.3.3.6 Sequencing on Illumina platform and data analysis

Sequencing was carried out by Edinburgh Genomics and sequence data were analyzed in collaboration with bioinformaticians in the Edinburgh University according to the algorithms described in section 5.2.2.

2.3.4 Generation of lentiviral constructs incorporating selected bovine TCR transcripts

2.3.4.1 Identification of TCR α and β chains expressed by epitope-specific CD8 T cell clones

RNA was isolated from cultured CD8 T cell clones using TRI-reagent (Sigma) according to the manufacturer's instructions. 5-10×10^6 cells were resuspended in approximately 100µl of standard cell culture medium, to which was added 1ml of TRI-reagent, mixed thoroughly by repeated pipetting and incubated at room temperature for 5min to allow complete dissociation of nuclear-protein complexes. 200µl chloroform was added and the tube was vortexed for at least 15 seconds and then incubated at room temperature for up to 15minutes. Following centrifugation for

15min at 12,000×g 4°C, the aqueous phase was transferred to a clean labelled 1.5ml tube and 500µl isopropanol added and mix. The tube was allowed to stand for 5-10min at room temperature and then centrifuged for 10min at 12,000×g, 4°C. The supernatant was discarded and the pellet resuspended in 1ml ethanol before centrifugation for 10minutes at 12000×g, 4°C. The ethanol supernatant was carefully removed and the pellet allowed to air dry until almost transparent. The pellet was resuspended in 10-25µl nuclease-free water and the tube allowed to stand at room temperature for 5-10min to allow RNA to fully dissolve. cDNAs were synthesized using the Promega® Reverse Transcription System (see section 2.3.3.3).

Specific primers (VB28F: 5'-CGCCAA GATCCGGGATTTG-3', JB3.2R: 5'-CAGCACAGTCAGCTTGGAAC-3') were designed and used to amplify TCR transcripts from CD8 T cell clones with the desired TRB clonotype. cDNAs prepared from $Tp2_{50-59}$ epitope-specific CD8 T cell clones were used for PCR amplification. PCR reactions in 0.2ml microcentrifuge tubes were set up by adding 10× PCR Buffer 2µl, 10µM VB28F 1µl, 10µM JB3.2R 1µl, template cDNA 2µl, Taq DNA Polymerase 0.1µl and nuclease-free water to give a final volume of 20µl. The reaction was gently mixed. The condition used in in the PCR reaction were: 95°C 5min; 5 cycles of 95°C 1min, 60°C 1min, 72°C 1min; 30 cycles of 95°C 30min, 60°C 1min, 72°C 1min; 72°C 5min. The PCR products were stored at 4°C and samples were run on gel electrophoresis to select clones with expected PCR product.

For selected CD8 T cell clones, the paired TCR α chain genes were analyzed using a panel of forward primers previously designed to amplify known bovine Vα gene subgroups or sets of genes within the subgroups and a reverse primer specific for bovine Cα gene. All primers are shown in Table 2-4. PCR conditions were as described above for TCR β chains.

Table 2-4 Primers for TCR α chain analysis

Vα	Forward	Reverse	Cα
1	5'-CAGGAAAAGGCGTTAAGCAG-3'	5'-GGGCTTCTCAGCTGGTACAC-3'	**TRAC**
2	5'-GGTCTCTTTGGAGGGAGCTG-3'		
3	5'-CAGCCAGAAGCTGAGGTC-3'		
4	5'-AGTGACCGTGCTCCTGAC-3'		
8.1	5'-GGCTCTC(A/C)TGACCCTGAACT-3'		
8.2	5'-TGATGCTTGAGATGCTCCTG-3'		
8.4	5'-CGGTCACATCAACGTCTCTG-3'		
8.5	5'-ATTCCAGAGGCCAGTCAGTG-3'		
9	5'-CTTCTCCAGGCTTAGTGACTG-3'		
10	5'-TGAGTGGCAAAAACCAAGTG-3'		
12	5'-CACAGTGGAGCAGAGTCCTG-3'		
13	5'-CTTGTGGCT(A/G)CAGCTGGAC-3'		
14	5'-AGGTGGTCGTGGCTTCACT-3'		
16	5'-CAAGAGCCCAGACAGTGACTC-3'		
17	5'-GAGAAGCTTCTGGCCCTG-3'		
18	5'-GTTGTTACCCT(C/T)CCCGAGAAG-3'		
19	5'-G(G/C)ATGTA(A/G)CCTTGAACTGTGC-3'		
20	5'-CCAGGAGGGGGACAGTCT-3'		
21	5'-GCCTGCTCATCCTTTGGTTA-3'		
22.1	5'-CTT(C/T)TGTTTGCCCAGGTTTG-3'		
22.2	5'-GGTGGAGCAGA(A/G)TCCTTCAG-3'		
23	5'-AAGTGACCAA(G/C)AGCAGGTGA-3'		
24	5'-CCCTTGCTGTGGGTTCAG-3'		
25	5'-G(G/C)ACCAGTGTTGATCTTA(C/T)GGA-3'		
26	5'-TCCATGGATT(A/G)T(G/C)CTGAAGG-3'		
27	5'-TGGTCTTTTGGATTCAACTGG-3'		
28	5'-ACAAAGAAGAGTCTTGCTGAGTC-3'		
29	5'-GCTTCAGTCTGACTGGGTG-3'		
33	5'-GGCGGACAAAGTTACTGAAGC-3'		
35	5'-GACATGTGTGAGTGCCCAAC-3'		
36	5'-CCCCATCTCTGATTGTCCAT-3'		
38	5'-CACAGTGACCCTGGACTGTA-3'		
41	5'-AGGAAGGAGACCTCGTCACA-3'		

Primers designed to amplify subfamily of bovine Vα genes are shown from 5' to 3' end. The reverse primer used to amplify bovine TCRα genes is specific for the constant gene of bovine TCR α chain.

2.3.4.2 Lentiviral constructs incorporating bovine TCR genes

Full-length cDNAs of determined TCR α and β transcripts were amplified from epitope-specific CD8 T cell clones. Forward primers complementary to the 5' untranslated regions (5' UTR) of selected Vα and Vβ genes were used in combination with reverse primers (TRAC and TRBC) complementary to 5'-end of Cα and Cβ genes respectively. The sequences of these α and β chain primers are shown in Table 2-5. The diagrammatic maps of mature TCR α and β transcripts and the location of designed primers are shown in Figure 2.3.

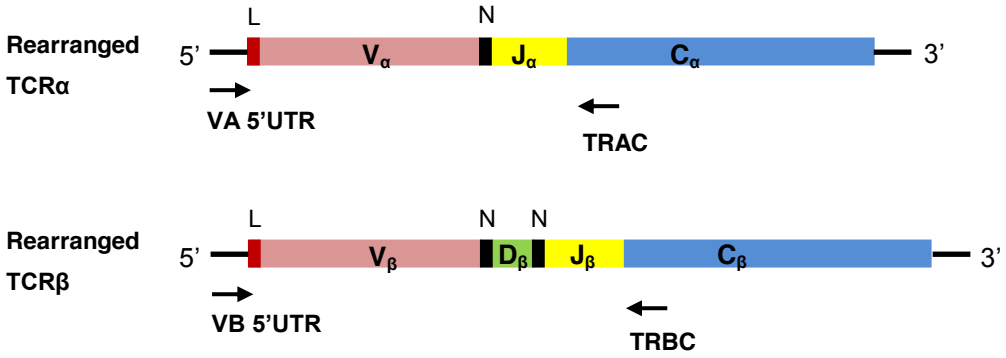


Figure 2.3 Illustration of primer design for obtaining full-length TCR α and β transcripts.
Mature αβT cells express TCR heterodimers composed of α chain (generated from germline VJ gene recombination) and β chain (generated from germline VDJ gene recombination). V, J and C gene segments of TCR α and β chains are shown in pink, yellow and blue respectively. Dβ gene segment is shown in green. Regions in black (N) represent non-germline nucleotide additions and deletions during gene rearrangement. Leader (L) sequences are shown in red. Forward primers were designed according to 5'-untranslated region (5'UTR). Reverse primers were complementary to the 5'-end of C gene segments.

The Phusion® high-fidelity PCR Kit (New England Biolabs) was used for PCR amplification according to the manufacturer's instructions (see section 2.3.3.4). The cDNAs encoding the TCR α and β chains were then combined into a single sequence (TCRβ-2A-TCRα) linked by an oligonucleotide sequence encoding the T2A "self-cleaving" peptide from *Thosea asigna* virus. Restriction enzyme sites XbaI (TATGAG) and XhoI (CTCGAG) were added to the 5' and 3' ends of the construct respectively for cloning into the lentiviral vector. The designed sequences were synthesized (Life Technologies) and then cloned into a vesicular stomatitis virus

glycoprotein (VSV-g)-pseudotyped HIV-based second-generation lentiviral vector pSxW.tCD34 (generously provided by Dr. John Bridgeman from Cardiff University, who also provided the packaging vectors pCMVΔ8.91 and pMD2.G). The gene expression is driven by a spleen focus-forming virus (SFFV) promoter. A human CD34 cDNA within the vector linked to the downstream of cloning site by a second 2A oligonucleotide (P2A from porcine teschovirus-1) and controlled by the same SFFV promoter was used as a reporter marker to detect successful gene transcription. The generated pSxW-TCR lentiviral transfer vector, as well as the two packaging plasmids, were transformed into XL-10 Gold Ultracompetent cells according to the manufacturer's instructions. Maxipreps of the three plasmids, prepared using the EndoFree Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions, were used for transfection of 293T cells for pseudotyped lentivirus production as described in section 2.2.12.

Table 2-5 Forward and reverse primers for cloning full-length cDNA of TCR α and β genes

Name	Sequences
VB28 5' UTR	5'-CATTTGACGCTACCATGTGC-3'
VA8 5' UTR	5'-ACATTCCTTCCTGCTCCTCA-3'
VA13 5'UTR	5'-GGAGACTGCAGGTCCACAAT-3'
VA26 5'UTR	5'-GGAKCAGWCRCAAKYCTGAGC(K=G,T; W=A,T; R=A,G; Y=C,T)-3'
TRBC	5'-GGAGATCTCTGCTTCCGAGGGTTC-3'
TRAC	5'-GGGCTTCTCAGCTGGTACAC-3'

Forward primers including VB28 5' UTR, VA8 5' UTR, VA13 5'UTR and VA26 5'UTR are specific for the 5' untranslated regions (5' UTR) of selected Vα and Vβ genes. Reverse primers TRBC and TRAC are specific for the constant genes of bovine TCR β and α chains.

Chapter 3: Epitope dominance hierarchies restricted by a bovine MHC I allele differ between homozygous and heterozygous animals

3.1 Introduction

The participation of CD8 T cells in immunity against *Theileria parva* has been clearly demonstrated [Pearson et al., 1979, Eugui and Emery, 1981, Morrison et al., 1987]. The protective ability of CD8 T cell mediated immune responses was illustrated by adoptive transfer of highly enriched CD8 T cells from immune to naïve twin calves [McKeever et al., 1994], although the involvement of responses by other cells can not be excluded [Taracha et al., 1997]. One feature of CD8 T cell responses against *T. parva* is strain specificity. When animals immunized with one parasite isolate are challenged with different parasite isolates, they show complete protection against challenge with the homologous isolate but variable patterns of protection against heterologous isolates [Irvin et al., 1983, Radley et al., 1975a]. Data from other studies suggest that incomplete cross-protection is due to a combination of polymorphism of the target antigens and immunodominance of the CD8 T cell responses [Taracha et al., 1995b]. Since the dominant antigens are determined by the presenting MHC class I (MHC I) alleles, both the antigen variants expressed by the parasite and the host MHC I genotypes influence the strain specificity of the CD8 T cell response against parasite infection [Goddeeris et al., 1990, Goddeeris et al., 1986].

Immunodominance has been observed as a general feature of CD8 T cell immunity against intracellular pathogens [Wilson and Hunter, 2008, Yewdell, 2006]. For individuals with a defined MHC I genotype, CD8 T cells tend to respond to only a few of the many 8-11-mer (generally) pathogen-derived peptides capable of binding to the expressed MHC I alleles. Among the limited number of epitopes recognised by the CD8 T cell response, some generate much stronger responses than others and this hierarchy is often reproducible between individuals of the same MHC I genotype. Such epitope dominance hierarchies have been observed in CD8 T cell responses against a number of viruses [Belz et al., 2000, Tscharke et al., 2005,

Brookes et al., 1995]. Identification of the most dominant epitopes was considered to be useful for the development of peptide-based vaccines, due to the immunogenicity of these epitopes and consistent dominance hierarchies in hosts with the respective MHC I genotypes [Chen et al., 2002, van der Most et al., 2003, Yu et al., 2002]. The mechanisms for immunodominance are not completely understood. However, results of research have suggested that a complex process involving factors determined by both the pathogen and the host determines immunodominance [Yewdell and Bennink, 1999, Yewdell, 2006, Akram and Inman, 2012]. Generally, these factors can be grouped into two broad categories: (1) those involved in peptide-MHC presentation, such as antigen abundance, peptide processing (i.e. proteasome digestion, TAP transport, peptide trimming) and MHC class I binding affinity [Tenzer et al., 2009, Kotturi et al., 2008, La Gruta et al., 2006b]; and (2) factors that determine activation, proliferation and differentiation of naïve T cells, such as frequency of epitope-specific naïve T cells and affinity of the T cell receptor for peptide-MHC I complexes [La Gruta et al., 2010, Denton et al., 2011, Chen et al., 2012]. The relative contribution of these factors to immunodominance appears to vary in different virus disease models [Kloverpris et al., 2013, Tenzer et al., 2009], illustrating the difficulty in drawing general conclusions on immunodominance.

Six *T. parva* antigens recognised by CD8 T cells from immune cattle were identified by Graham et al. (2006) and subsequent work defined nine epitopes in these antigens, together with their MHC I restriction elements [Graham et al., 2006, Graham et al., 2008]. Highly dominant responses to two of these *T. parva* antigens, Tp2 and Tp1, were demonstrated by clonal analysis of *in vitro*-generated CD8 T cell lines from pairs of immune animals homozygous for the BoLA-A10 (abbreviated as A10) and A18 MHC I haplotypes respectively [MacHugh et al., 2009]. Screening of the CD8 T cells from these animals against 5 of the *T. parva* antigens showed that in each case they only recognised one of the antigens, both A18 animals recognising Tp1 and both A10 animals recognising Tp2. Moreover, over 50% of the CD8 T cell response of each animal was specific for the respective antigen. Two epitopes, $Tp2_{49-59}$ (KSSHGMGKVGK) and $Tp2_{98-106}$ (QSLVCVLMK), were initially identified in the Tp2 antigen and both were determined to be presented by the same A10 MHC I

allele BoLA 2*01201 [Graham et al., 2008]. The response to the $Tp2_{49-59}$ epitope was highly dominant in both animals accounting for 59-74% of the response, with a small component (2-4%) responding to the $Tp2_{98-106}$ epitope [MacHugh et al., 2009]. Later studies revealed that the population of CD8 T cells that reacted with the $Tp2_{49-59}$ peptide was actually composed of 2 distinct populations – one specific for $Tp2_{49-59}$ and another specific for $Tp2_{50-59}$ (SSHGMGKVGK), which is also presented by BoLA 2*01201 (Connelley et al., - in preparation).

To enable direct detection and quantification of epitope-specific CD8 T cell responses both *in vitro* and *in vivo*, peptide-MHC I (pMHC I) tetramers incorporating each of the three BoLA 2*01201-restricted Tp2 epitopes were generated. Soluble pMHC I tetramers were initially introduced in 1996 and have been used extensively for the study of antigen-specific CD8 T cell responses [Altman et al., 1996]. Comparing with techniques such as cytotoxicity and IFN-γ ELISPOT, which depend on the functional capacity of CD8 T cells, pMHC I tetramers requires only the expression of cognate TCR to define antigen specificity of responding CD8 T cells [Doherty, 2011]. Moreover, fluorochrome-conjugated pMHC I tetramers allows rapid quantification of epitope-specific CD8 T cell populations, which is less labour- and time-consuming in comparison to functional assays [Klenerman et al., 2002].

The previous data demonstrating the dominant status of CD8 T cells specific for the $Tp2_{49-59}$ epitope were based on two BoLA-A10 homozygous cattle. In addition, the proportion of CD8 T cells that were specific for the distinct $Tp2_{49-59}$ and $Tp2_{50-59}$ epitopes had not been determined. The work described in this chapter used pMHC I tetramer staining to determine the relative frequencies of CD8 T cells specific for the 3 defined Tp2 epitopes in 11 A10 cattle immunised with *T. parva*, including both homozygous and heterozygous animals. The results showed that epitope dominance hierarchies differ markedly between homozygous and heterozygous animals.

3.2 Materials and methods

3.2.1 Animals

Animals used in this chapter are listed in section 2.1.

3.2.2 *In vitro* generation of CD8 T cell enriched cell lines

Details of the procedure for generating CD8 T cell lines are provided in section 2.2.5. Briefly, PBMC isolated from *T. parva*-immunized A10-homozygous and -heterozygous animals were stimulated with gamma-irradiated *T. parva* (Muguga isolate) infected cells (TpM cells), either from the autologous animal, or MHC-matched or A10-homozygous animals. After a second stimulation, CD4 and γδ T cells were depleted from cell cultures. Purified CD8 T cells were maintained by stimulation at 7-14 day intervals with irradiated TpM cells, in the presence of rIL2 at a final concentration 100U/ml. Cell lines obtained after the third stimulation were cryopreserved.

3.2.3 Generation of T cell cultures from purified CD8 T cells

1×10^8 PBMC were pelleted by centrifugation at 400×g for 5min at 4°C and re-suspended in 5ml of IL-A105 hybridoma culture supernatant (antibody specific for CD8). After incubation for 10min at room temperature, cells were washed twice with PBS and centrifuged at 400×g for 5min at 4°C. After the second wash, fluid above the cell pellet was removed completely. Pelleted cells were re-suspended directly with goat anti-mouse IgG micro-beads (100µl per 1×10^8 cells) and incubated for 10min at room temperature. Cells were washed twice with PBS and re-suspended in 2ml MACS buffer (PBS with 2mM EDTA and 0.5% BSA). The MS column was washed with 1ml MACS buffer before the addition of the prepared cells. The column was washed with 1ml of MACS buffer and the bound CD8 T cells then eluted in 2ml SCM into a 15ml tube containing 5ml SCM. After centrifugation at 400×g for 5min, pelleted cells were re-suspended in appropriate SCM for subsequent use.

3.2.4 Tetramer staining of *in vitro* CD8 T cell cultures

In vitro generated CD8 T cell-enriched cell lines were harvested and re-suspended in SCM at a density of 1×10^7/ml. Aliquots of 100µl cell suspension were distributed in wells of 96-well round-bottom well plates, centrifuged briefly and re-suspended in 50µl of 20nM pMHC I tetramers. PE labelled pMHC I 2*01201 tetramers that incorporate the three defined epitopes derived from the Tp2 antigen ($Tp2_{98\text{-}106}$, $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$) were generated by Pierre van der Bruggen and Didier Colau from Ludwig Institute for Cancer Research, Brussels, Belgium, according to the method described previously [Lonchay et al., 2004]. After incubation with pMHC I tetramers at 4°C for 30min in the dark, cells were washed twice with FACS medium and re-suspended in 200µl FACS medium for analysis using a BD FACSCalibur cell analyser. Negative controls were incubated with FACS medium instead of tetramers.

3.2.5 Peptide stimulation of CD8 T cells

For peptide stimulation, an A10-homozygous *Theileria annulata*-infected cell line (TA) 592TA was used as an antigen-presenting cell to assay T cell recognition of A10-restricted epitopes from *T. parva*. Previous experiments had shown that there was no antigenic cross-reactivity of *T. parva*-specific CD8 T cells with *T. annulata*. Peptides for the three defined Tp2 epitopes were produced by Pepscan Systems (Lelystad, Netherland). Target cells were re-suspended in SCM at 1×10^7/ml and incubated for 1 hour with 1µg/ml peptide at 37°C 5% CO_2. Cells were washed once with PBS after incubation and exposed to 60Gy of gamma irradiation. In each well of a 24-well plate, 2×10^5 CD8 T cells were co-cultured with 1×10^6 peptide-loaded 592TA cells for *in vitro* stimulation.

3.2.6 *Ex vivo* detection of epitope-specific CD8 T cells

PBMC from parasite challenged animals were isolated at chosen time points and then stained to enable two-colour flow cytometric analysis. Briefly, PBMC were initially stained with pMHC I tetramers at a final concentration of 20nM. After 30min incubation at 4°C, cells were washed twice and stained with 50µl of primary antibody cocktail, including monoclonal antibodies specific for CD4 (IL-A12 IgG2a)

and γδ (GB21A, IgG2b) T cells, B cells (CD21, IgG1), NK cells (Gr13, IgG2b) and monocytes (IL-A24, IgG1). All mAbs had been titrated using PBMC to determine optimal dilutions prior to the experiment and it had been demonstrated that this combination of antibodies left $CD8^+$ $\gamma\delta^-$ (i.e. $\alpha\beta^+$) CD8 T cells as the only unstained population in PBMC. Cells were incubated at 4°C for 30min, washed three times in FACS medium and stained with 50µl of 1µg/ml Alexa Flour 647 labelled goat anti-mouse IgG (H+L) antibody (Life technologies, Paisley, UK). Following incubation at 4°C for 30min, cells were washed three times in FACS medium and then re-suspended in 200µl FACS medium. Tenminutes before FACS analysis, SYTOX Red Dead Cell Stain (Life technologies, Paisley, UK) was added at 1:1000 dilution according to the manufacture's recommendation.

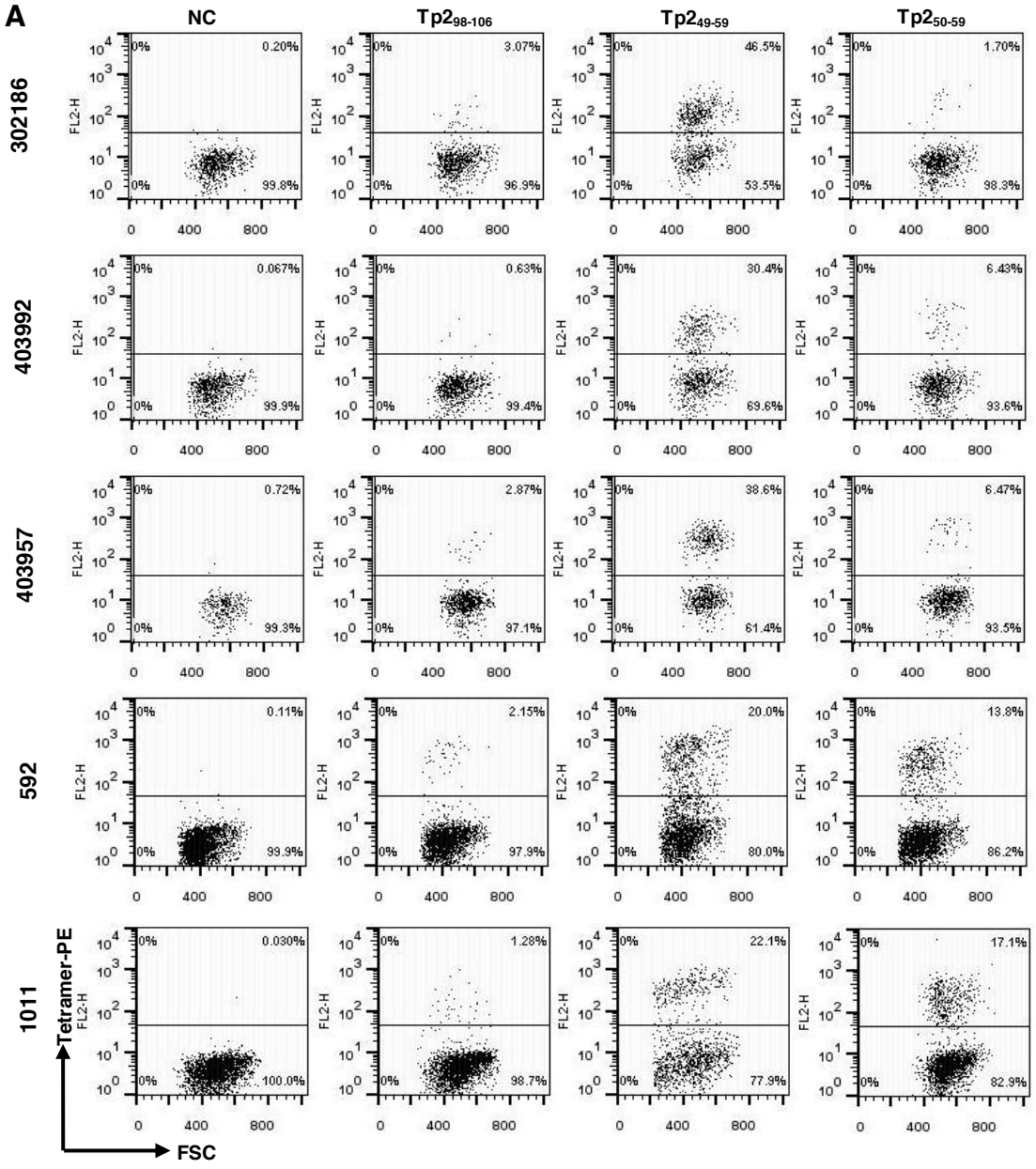
3.3 Results

3.3.1 Epitope-specificity of A10-homozygous animals shows consistent dominance hierarchies

The antigen specificity of CD8 T cell responses were initially analysed in A10-homozygous animals, which was aiming to determine whether Tp2 is consistently a dominant antigen and define the immunodominance hierarchies of the three defined BoLA 2*01201-restricted Tp2 epitopes.

The results obtained by staining *in vitro* generated CD8 T cell lines from five immunized A10-homozygous cattle with $Tp2_{98-106}$, $Tp2_{49-59}$ and $Tp2_{50-59}$ pMHC I tetramers are shown in Figure 3.1. Cultures from all five animals, which had undergone 3 stimulations (animals 302186, 403992 and 403957), or ≥5 stimulations (animals 592 and 1011) were found to contain 20-46% CD8 T cells specific for the $Tp2_{49-59}$ epitope with smaller numbers of CD8 T cells specific for the $Tp2_{98-106}$ epitope (0.7-3%). The percentages of CD8 T cells specific for the $Tp2_{50-59}$ epitope showed greater variation, ranging from 1.5% to 17% in the five animals. Nevertheless, overall the immunodominance hierarchy of the three defined Tp2 epitopes showed a similar profile in all five animals.

The results clearly showed that $Tp2_{49-59}$ was consistently the most dominant epitope in A10-homozygous animals with $Tp2_{50-59}$ and $Tp2_{98-106}$ being subdominant.


A
NC
Tp2 98-106
Tp2 49-59
Tp2 50-59
302186
403992
403957
592
1011
0.20%
3.07%
46.5%
1.70%
99.8%
96.9%
53.5%
98.3%
0.067%
0.63%
30.4%
6.43%
99.9%
99.4%
69.6%
93.6%
0.72%
2.87%
38.6%
6.47%
99.3%
97.1%
61.4%
93.5%
0.11%
2.15%
20.0%
13.8%
99.9%
97.9%
80.0%
86.2%
0.030%
1.28%
22.1%
17.1%
100.0%
98.7%
77.9%
82.9%
FL2-H
Tetramer-PE
FSC

B

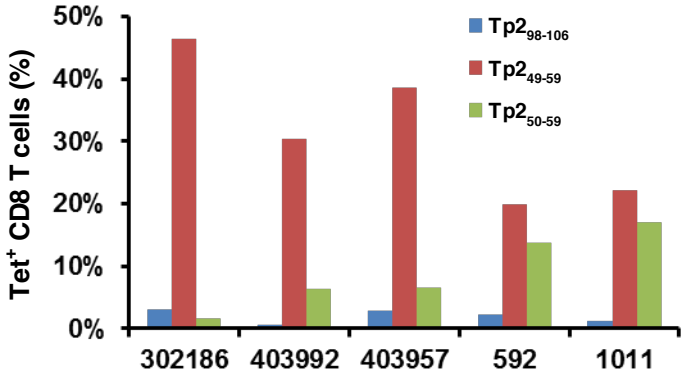


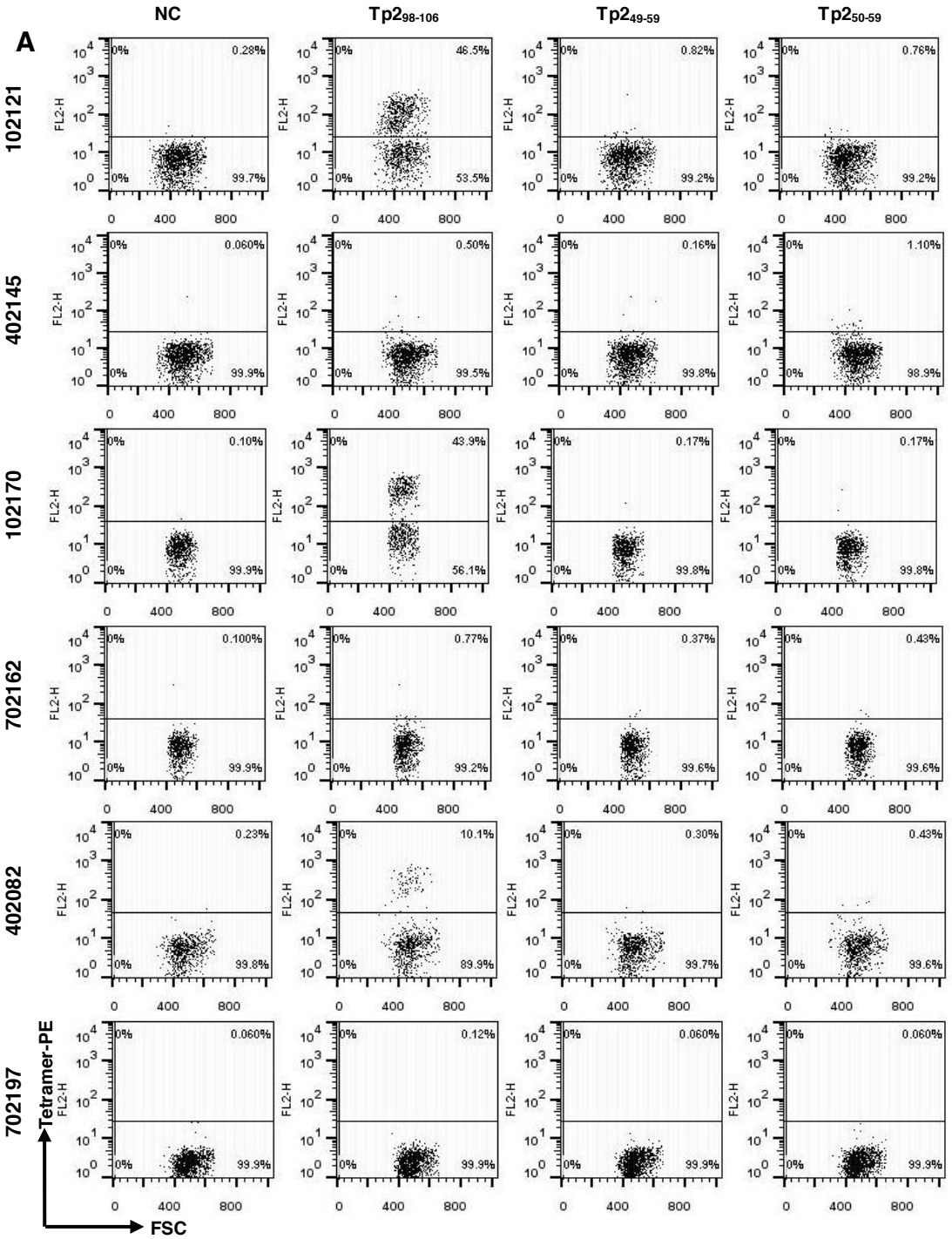
Figure 3.1 Epitope dominance profile in BoLA-A10 homozygous cattle. A, Flow cytometric analysis results of pMHC I tetramer ($Tp2_{98-106}$, $Tp2_{49-59}$ and $Tp2_{50-59}$/2*01201) staining of CD8 T cell lines generated from 5 *T. parva* immunized A10-homozygous animals. Animal numbers are labelled and shown on the left. Gated lymphocytes are used for analyses. Gating strategy is not shown. X axis, forward scatter (FSC); Y axis, Phycoerythrin (PE) -conjugated pMHC I tetramer (Tetramer-PE). Percentages of tetramer positive cells are shown in the upper right corner of each plot. B, Summary of Tp2 epitope frequency in the five A10-homozygous animals.

3.3.2 The Tp2 epitope dominance hierarchy in A10-heterozygous animals is different

To investigate CD8 T cell epitope specificity and epitope dominance hierarchies of Tp2-specific responses in A10-heterozygous cattle, CD8 T cell lines were generated from 6 A10-heterozygous cattle following three *in vitro* stimulations with autologous parasitized cells.

The *T. parva* (Muguga)-immunized animals comprise three pairs of MHC I-matched A10-heterozygous animals expressing the A11, A12 and N5 MHC I haplotypes. Tp2 epitope-specific CD8 T cells were detected in CD8 T cell lines from only three of the six animals (one from each MHC I-matched pair). These cells only recognised the $Tp2_{98-106}$ epitope (Figure 3.2). The percentages of $Tp2_{98-106}$-specific CD8 T cells were 46.5%, 43.9% and 10.1% for animals 102121 (A10/A12), 102170 (A10/N5) and 402082 (A10/A11), respectively. CD8 T cells specific for $Tp2_{49-59}$ and $Tp2_{50-59}$ epitopes were not detected in any of the six CD8 T cell lines.

The results demonstrated that in contrast to A10-homozygous animals, A10-heterozygous animals i) do not all generate a detectable CD8 T cell response to Tp2 and ii) when responses are observed it is only against the $Tp2_{98-106}$ epitope.



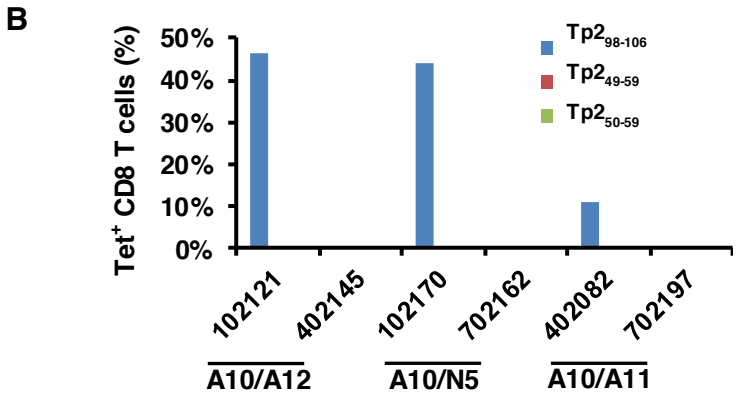


Figure 3.2 Epitope dominance profile in BoLA-A10 heterozygous cattle. A, flow cytomeric analysis results of pMHC I tetramer ($Tp2_{98-106}$, $Tp2_{49-59}$ and $Tp2_{50-59}$/2*01201) staining of CD8 T cell lines generated from 6 *T. parva* immunized A10-heterozygous animals. Animal numbers are labelled and shown on the left. Gated lymphocytes are used for analyses. Gating strategy is not shown. X axis, forward scatter (FSC); Y axis, Phycoerythrin (PE)-conjugated pMHC I tetramer (Tetramer-PE). Percentages of tetramer positive cells are shown in the upper right corner of each plot. B, summary of Tp2 epitope frequency in the 6 A10-heterozygous animals.

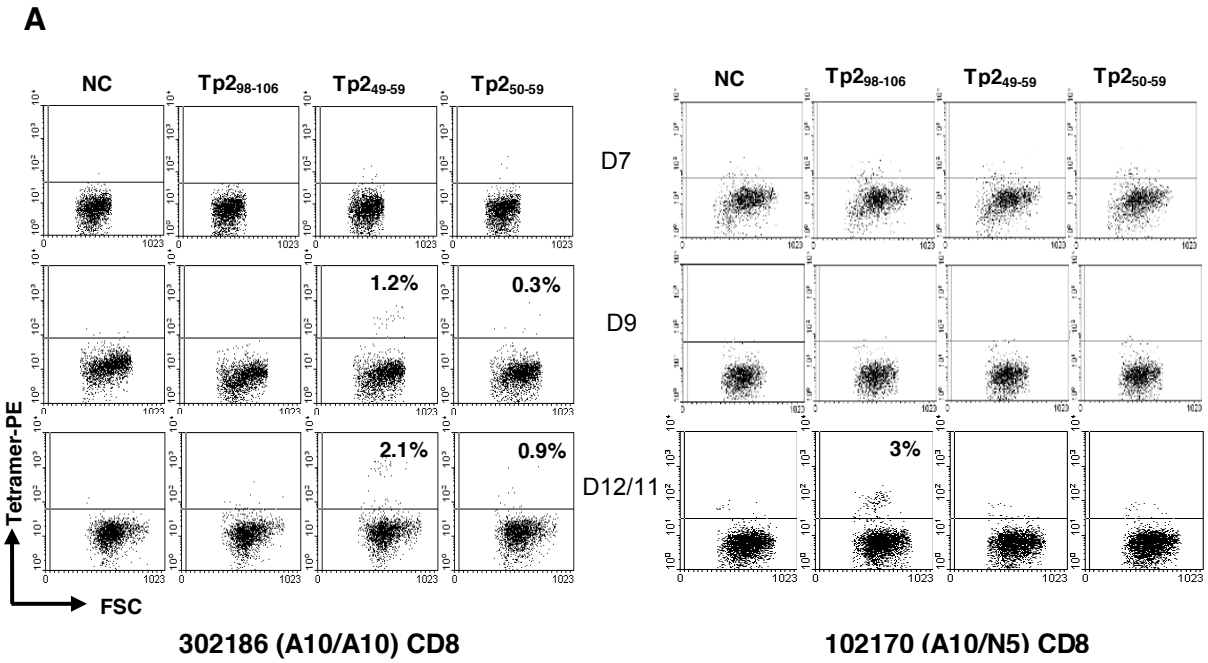
3.3.3 The specificity of CD8 T cells stimulated once *ex vivo* is similar to that of T cell lines

Since the results obtained for the A10-heterozygous animal were based on analyses of CD8 T cell lines generated following 3 *in vitro* stimulations, one concern was that the inconsistent detection of responses to the $Tp2_{98-106}$ epitope and the absence of $Tp2_{49-59}$ and $Tp2_{50-59}$ epitope-specific responses may have arisen as a result of changes in composition of CD8 T cell lines following *in vitro* passage as has been reported in other models (Koning et al., 2014, Belyakov et al., 2001). In order to detect epitope-specific CD8 T cells at the earliest time point *in vitro*, primary CD8 T cell cultures generated by a single *in vitro* stimulation of purified CD8 T cells with irradiated parasitized cells were generated. Tetramer staining was applied on days 7, 9 and 11 (or 12) after primary stimulation of purified CD8 T cells from three A10-homozygous and all six A10-heterozygous animals. As shown in Figure 3.3, the numbers and intensity of staining of positive cells were optimal on day 9 for homozygous cattle and day 11 for heterozygous cattle.

The $Tp2_{49-59}$ epitope showed consistent dominance in the two homozygous animals (302186 and 403992) during the period of detection (Figure 3.4 A and B). The percentage of detectable $Tp2_{49-59}$ epitope-specific CD8 T cells increased from 1.3% on day 9 to 6.8% on day 13 for 302186, while for 403992, they showed high percentages (7-12%) with the peak on day 12. Only small numbers of $Tp2_{50-59}$ epitope-specific CD8 T cells were detected on day 12 for both animals (1% for 302186 and 2% for 403992). For animal 403957, the relative dominance of epitopes $Tp2_{49-59}$ and $Tp2_{50-59}$ had changed during *in vitro* culture. As shown in Figure 3.4C, percentages of CD8 T cells specific for the $Tp2_{49-59}$ epitope on day 9 and 12 (2.8% and 4.2% respectively) were smaller than those for the $Tp2_{50-59}$ epitope (4.2% on day 9 and 7.6% on day 12). However, on day 13, $Tp2_{49-59}$ epitope-specific CD8 T cells showed higher percentage (5.2%) than that for $Tp2_{50-59}$ epitope (4.4%). Low percentages (0.5-0.95%) of $Tp2_{98-106}$-specific CD8 T cells were detected for the three animals on day 12 or 13.

The epitope specificity of the CD8 T cells from A10-heterozygous animals showed the same profile as in cultures subjected to 3 stimulations. $Tp2_{98-106}$ epitope-specific CD8 T cells ranging from 0.7% to 13.7% (day 11 of stimulation) were detected from the three animals that had $Tp2_{98-106}$-specific responses in cultures following 3 *in vitro* stimulations, while CD8 T cells specific for $Tp2_{49-59}$ and $Tp2_{50-59}$ epitopes were not detected in any of the cultures.

These findings indicate that repeated *in vitro* stimulations did not cause significant changes in the epitope specificities elicited in CD8 T cell lines from A10 animals immunised with *T. parva*.



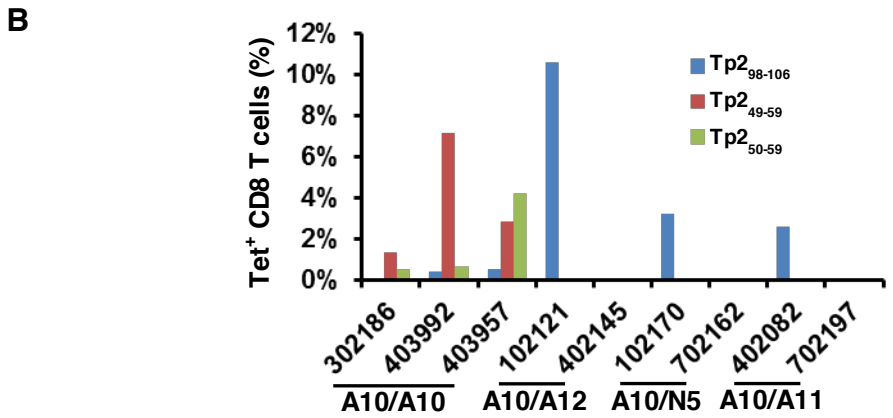


Figure 3.3 Epitope dominance in primary cultured CD8 T cell lines. To reduce *in vitro* expansion, purified CD8 T cells from immunized animals were stimulated once with autologous TpM. A, The generated CD8 T cell lines were stained with pMHC I tetramers at chosen time points. The optimal detection of epitope-specific CD8 T cells was on day 9 or 11 of stimulation. B, Percentages of epitope-specific CD8 T cells detected at the optimal time point (i.e. highest frequency of tetramer positive cells) showed $Tp2_{49-59}$ epitope dominance in A10-homozygous cattle (except animal 403957) and $Tp2_{98-106}$ epitope dominance in A10-heterozygous cattle.

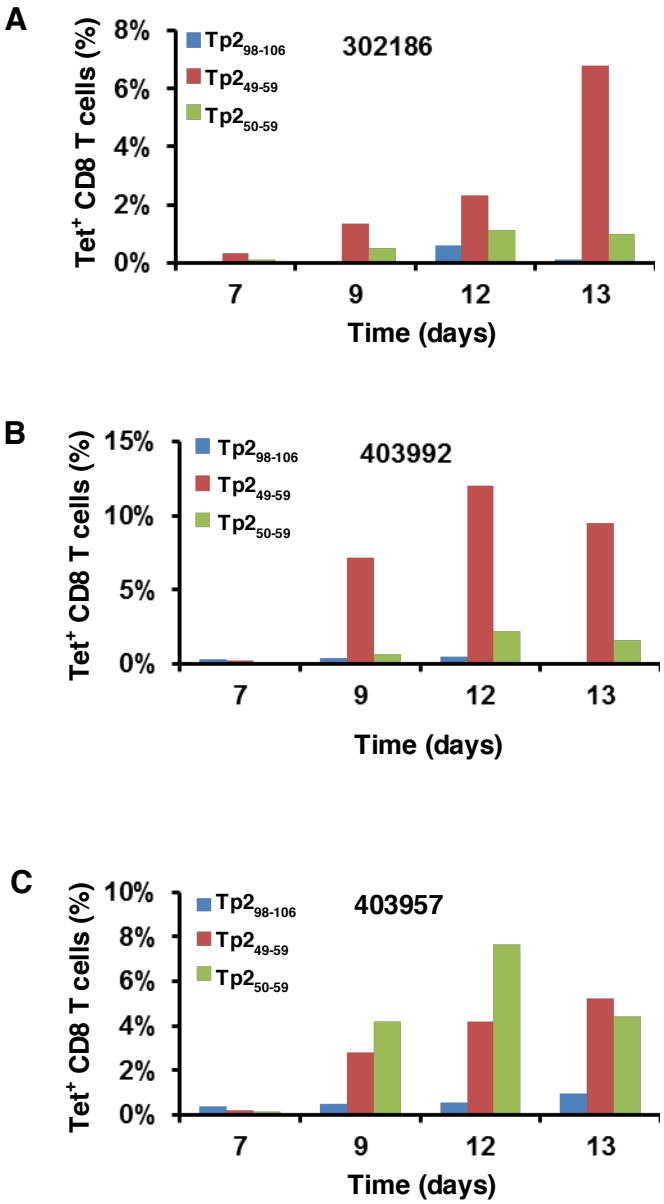


Figure 3.4 Kinetics of Tp2 epitope-specific responses in primary CD8 T cell cultures of A10-homozygous animals.

A and B, The responses against $Tp2_{49-59}$ was dominant in animals 302186 and 403992 throughout the time-course of the analysis. C, In animal 403957, the percentages of CD8 T cells specific for the $Tp2_{50-59}$ epitope were higher than that of $Tp2_{49-59}$ epitope upto day 12 post-stimulation but as a consequence of a rapid reduction in the frequency of $Tp2_{50-59}$ epitope-specific CD8 T cells on day 13 (from 7.6% on day 12 to 4.4% on day 13), the $Tp2_{49-59}$-specific response became dominant.

3.3.4 Epitope specificity variation between paired MHC I identical heterozygous cattle was not due to deficiencies in the stimulator cells

In the above results, responses to the Tp2 antigen were consistently detected in only one of each pair of MHC I identical A10-heterozygous cattle. The possible deficiency of the autologous stimulator cell lines in the A10-heterozygous animals that did not show a Tp2-specific response was investigated by comparing CD8 T cell cultures generated by stimulation of purified CD8 T cells from each animal with the autologous infected cells or the MHC I-matched infected cells. MHC I-mismatched TpM cell lines were used as a negative control. The results of tetramer staining of the generated CD8 T cell primary cultures from the six A10-heterozygous animals are shown in Figure 3.5 A and B. For the A10-heterozygous cattle that showed Tp2-specific responses, $Tp2_{98\text{-}106}$-specific CD8 T cells ranged from 2% to 11% were detected from primary CD8 T cell cultures subjected to autologous and MHC I-matched stimulations, but not from MHC I-mismatched stimulations. For the A10-heterozygous cattle that did not have a Tp2-specific response, Tp2 epitope-specific CD8 T cells were still not detectable in any of the primary CD8 T cell cultures subjected to autologous or MHC I-matched stimulations. These results suggested that the failure to detect Tp2-specific CD8 T cells was not due the deficiencies in the stimulator cells, but rather reflected genuine differences between the pairs of MHC I identical animals in the antigenic specificity of the CD8 T cell response.

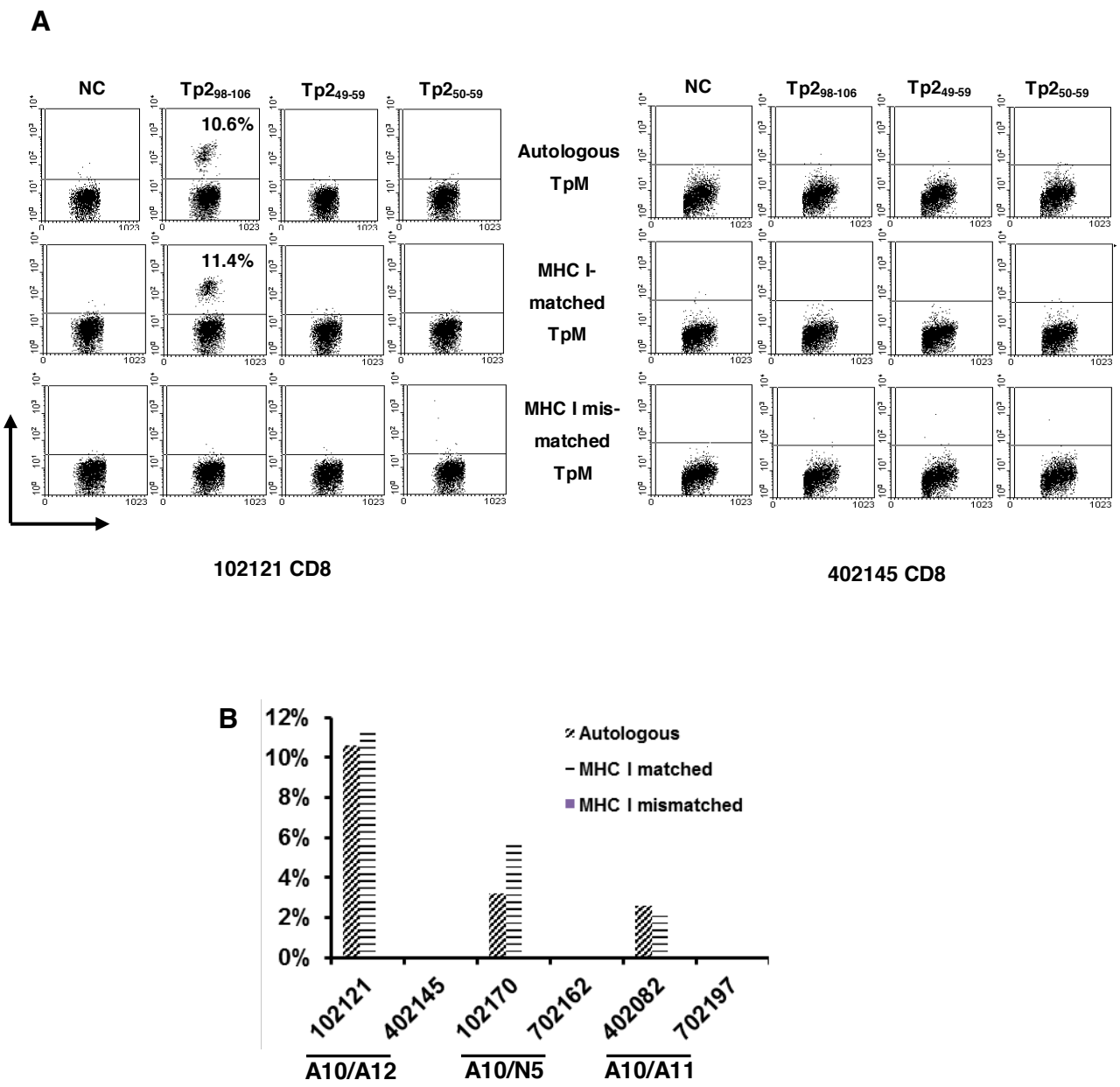


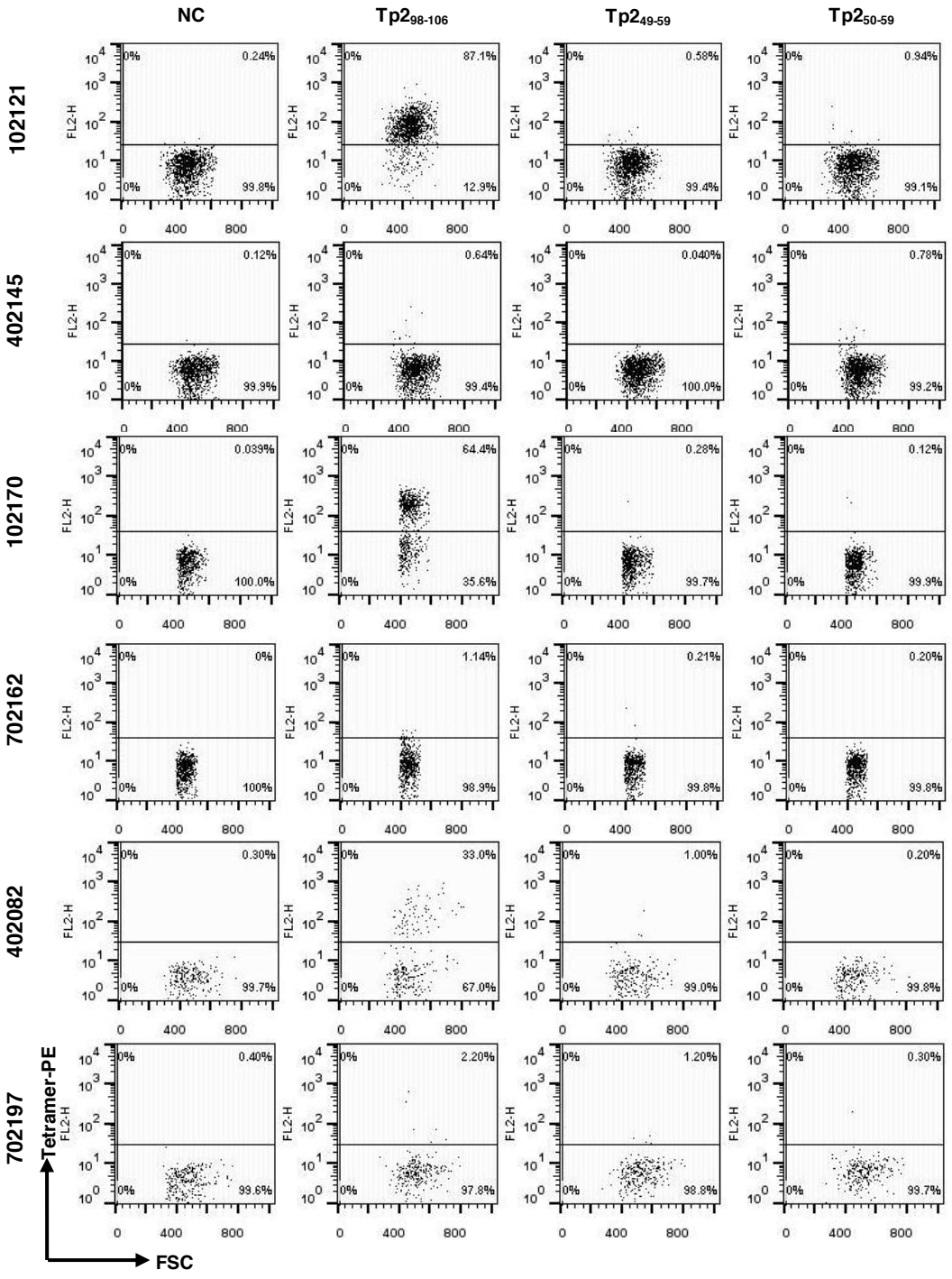
Figure 3.5 $Tp2_{98-106}$ epitope specificities of A10-heterozygous CD8 T cell lines stimulated with different TpM cell lines.

A, Optimal detection of $Tp2_{98-106}$ specific CD8 T cells from one pair of MHC I matched heterozygous animals 102121 and 402145. B, Overall quantitative analysis of $Tp2_{98-106}$ epitope-specific CD8 T cells in the 6 A10 heterozygous cell lines stimulated with different stimulators. MHC I-matched TpM represents the TpM cell line derived from the other animal within each pair of MHC I-matched A10-heterozygous cattle.

3.3.5 The same epitope specificity was detected for heterozygous CD8 T cell lines after homozygous TpM stimulation

In the A10-heterozygous animals, the failure to detect a Tp2-specific response in some animals may be due to the presence of a strong CD8 T cell response restricted by the non-A10 MHC I haplotype, thus masking detection of a Tp2-specific response. To investigate this possibility, CD8 T cell cultures were generated from the 6 A10-heterozygous cattle by stimulation with irradiated *T. parva*-infected A10-homozygous cells (592 TpM). CD8 T cell lines were subjected to 3 stimulations to maximise the chance of detecting selectively expanded Tp2-specific CD8 T cells. The Tp2 antigen specificities detected in these CD8 T cell cultures are shown in Figure 3.6 A and B. The three cattle in which $Tp2_{98\text{-}106}$-specific responses had been observed previously again exhibited $Tp2_{98\text{-}106}$-specific CD8 T cell responses, with 33-87% cells positive for the tetramer. Similarly, for the other 3 animals, in which $Tp2_{98\text{-}106}$-specific responses had not been observed previously, the use of the A10-homozygous TpM for stimulation hadn't modified the Tp2-responses and there was no evidence of any $Tp2_{98\text{-}106}$-specific responses. Moreover, in none of the six A10-heterozygous animals were $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$-specific T cells detected.

A


NC
Tp2$_{98-106}$
Tp2$_{49-59}$
Tp2$_{50-59}$
102121
402145
102170
702162
402082
702197
Tetramer-PE
FSC
FL2-H
0%
0.24%
99.8%
87.1%
12.9%
0.58%
99.4%
0.94%
99.1%
0.12%
99.9%
0.64%
99.4%
0.040%
100.0%
0.78%
99.2%
0.039%
100.0%
64.4%
35.6%
0.28%
99.7%
0.12%
99.9%
0%
100%
1.14%
98.9%
0.21%
99.8%
0.20%
99.8%
0.30%
99.7%
33.0%
67.0%
1.00%
99.0%
0.20%
99.8%
0.40%
99.6%
2.20%
97.8%
1.20%
98.8%
0.30%
99.7%
0
400
800

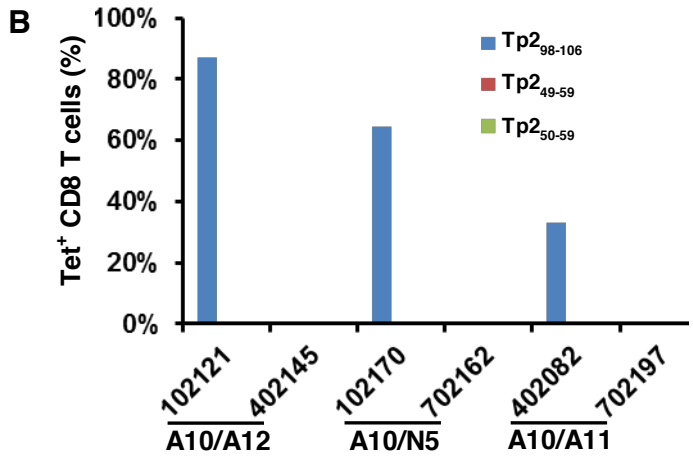


Figure 3.6 Tp2-specific responses of A10-heterozygous CD8 T cell cultures generated by stimulation with A10-homozygous TpM stimulator cells

A, Flow cytometric analysis results of pMHC I tetramer ($Tp2_{98-106}$, $Tp2_{49-59}$ and $Tp2_{50-59}$/2*01201) staining of CD8 T cell lines generated from 6 *T. parva* immunized A10-heterozygous animals subjected to A10-homozygous TpM stimulation. Animal numbers are labelled and shown on the left. Gated lymphocytes are used for analyses. Gating strategy is not shown. X axis, forward scatter (FSC); Y axis, Phycoerythrin (PE) -conjugated pMHC I tetramer (Tetramer-PE). Percentages of tetramer positive cells are shown in the upper right corner of each plot. B, Summary of Tp2 epitope-specific CD8 T cell responses in the six A10-heterozygous CD8 T cell lines stimulated with A10-homozygous TpM.

3.3.6 *Ex vivo* detection confirmed the differences in antigen specificities between homozygous and heterozygous animals

Epitope specificity and dominance hierarchies of the three defined Tp2 epitopes were examined directly during the *in vivo* response to *T. parva* in both BoLA-A10 homozygous and heterozygous animals. Four immunized A10 animals consisting of two homozygous and two heterozygous animals were challenged with the same parasite strain used for immunization. Epitope specificities of CD8 T cells were tracked by pMHC I tetramer staining of freshly isolated PBMC at different time points after parasite challenge. Figure 3.7 illustrates the gating strategy for detection of epitope-specific CD8 T cells from PBMC. Negative selection of CD8 T cells by staining with a panel of antibodies specific for CD4 T cells, γδ T cells, NK cells, B cells and monocytes (as described in section 3.2.6) allows enumeration of tetramer-stained CD8 T cells within the unstained population, avoiding interference of tetramer staining by anti-CD8 antibody [Wooldridge et al., 2006, Wooldridge et al., 2003]. Preliminary results confirmed that negatively stained cells as gated in Figure 3.7B represented CD8 T cells in PBMC.

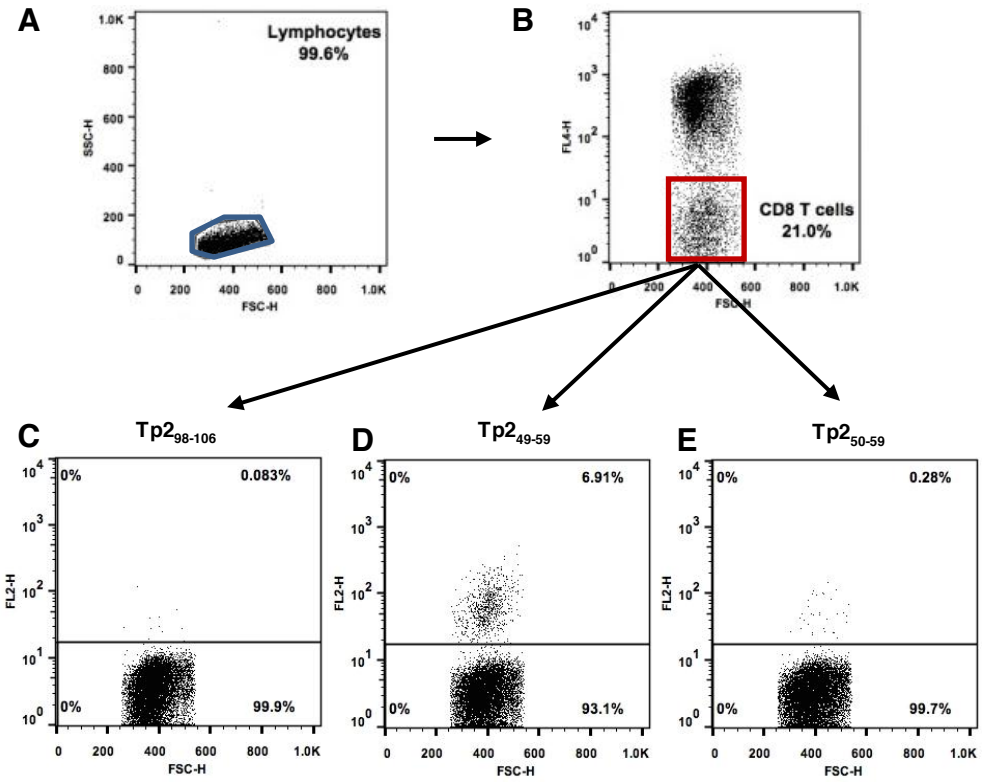


Figure 3.7 Gating strategy for *ex vivo* detection of epitope-specific CD8 T cells in PBMC. The blue gate in A represents lymphocytes which are separated into two populations as shown in B. Cells in red gate represents $CD8^+$ T cells which are negative for antibodies specific for CD4 T cells, γδ T cells, NK cells, B cells and monocytes. C, D, E showed tetramer positive CD8 T cells for the three defined Tp2 epitopes respectively.

The kinetics of the epitope-specific CD8 T cell responses in both homozygous and heterozygous animals, as detected by pMHC I tetramer staining, are shown in Figure 3.8. Antigen-specific CD8 T cells were first detectable between day 10 and day 12 after challenge. For the two A10-homozygous animals 302186 and 403992, $Tp2_{49\text{-}59}$ epitope-specific CD8 T cells reached peak levels (7.5% of CD8 T cells for animal 302186 and 0.5% of CD8 T cells for animal 403992) on day 14 after parasite challenge. CD8 T cells specific for the subdominant epitope $Tp2_{98\text{-}106}$ were not detectable during challenge. Due to the low supply of $Tp2_{50\text{-}59}$ epitope-specific tetramer, staining for this epitope specificity was not carried out until day 13 of challenge. CD8 T cells specific for the $Tp2_{50\text{-}59}$ epitope were only detected in animal 302186, at a level of 0.28% of CD8 T cells on day 13 (Figure 3.7E). For A10-

heterozygous animals 102121 and 402082, CD8 T cells specific for the $Tp2_{98-106}$ epitope were detected reaching peak levels of 1.7% and 2.2% pMHC I tetramer-positive cells (out of CD8 T cell populations) respectively on day 13 after challenge. CD8 T cells specific for epitopes $Tp2_{49-59}$ and $Tp2_{50-59}$ were not detected in the A10-heterozygous animals (data not shown). Overall, *ex vivo* tetramer staining confirmed the dominance of the $Tp2_{49-59}$ epitope in A10-homozygous cattle and the $Tp2_{98-106}$ epitope dominance in A10-heterozygous cattle, consistent with the *in vitro* observations.

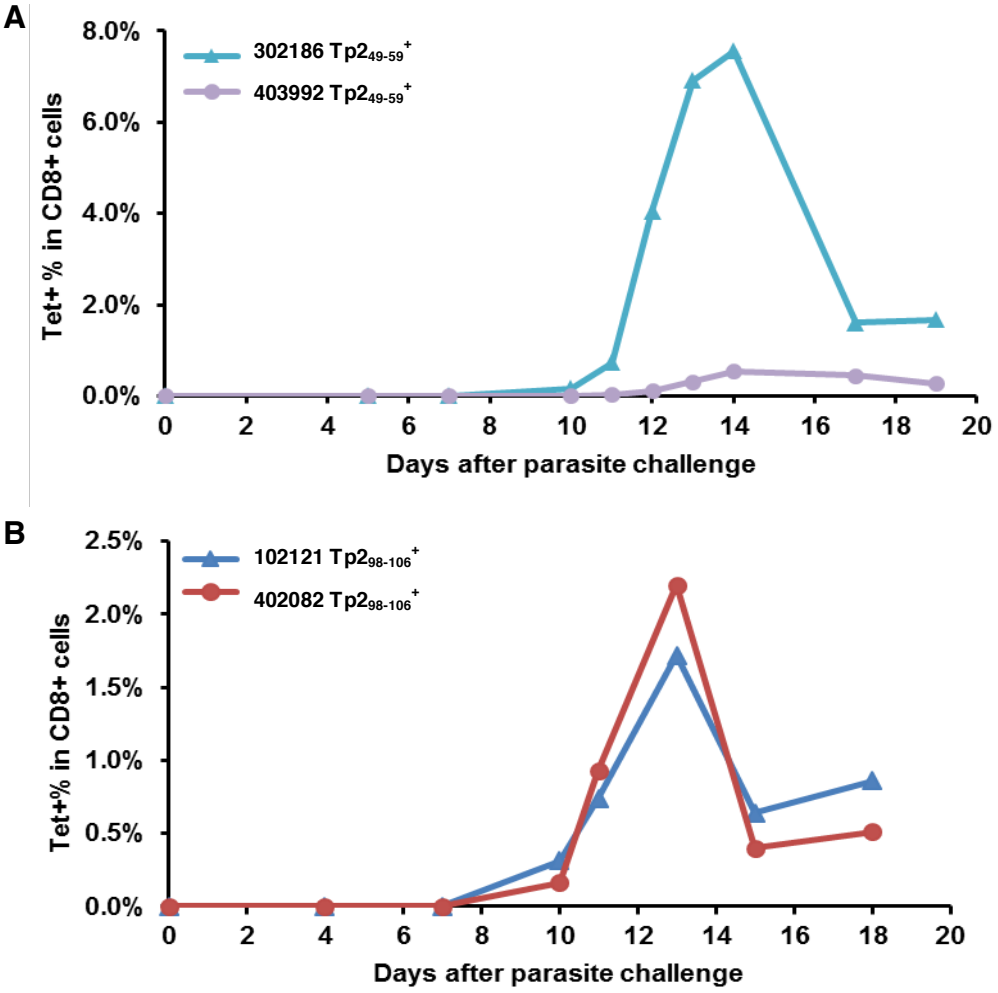


Figure 3.8 *Ex vivo* quantitative analysis of Tp2 epitope dominant CD8 T cell responses in A10-homozygous and -heterozygous animals.

A, Percentages of CD8 T cells specific for the $Tp2_{49-59}$ epitope in two A10-homozygous animals 302186 and 403992 after parasite challenge. B, Percentages of CD8 T cells specific for the $Tp2_{98-106}$ epitope in two A10-heterozygous animals 102121 and 402082 after parasite challenge.

3.3.7 Initial attempt to detect antigen-specific CD8 T cells from negative A10 heterozygous animals

pMHC I tetramers have been widely used for quantification of antigen-specific CD8 T cell responses both *in vitro* and *in vivo* [Altman et al., 1996, Flynn et al., 1998, La Gruta et al., 2010]. However, accurate identification of low frequency events is difficult, requiring extra care to distinguish true antigen-specific CD8 T cells from unspecific background [Chattopadhyay et al., 2008]. Cultures from 3 of the A10-heterozygous animals (402145, 702162 and 702197) were defined as negative for the $Tp2_{98\text{-}106}$ epitope specificity because discrete pMHC I tetramer-positive populations could not be identified with certainty. However, as shown in Figure 3.2 and 3.6, small numbers of positive signals were sometimes detected in these animals (e.g., 702197 CD8 T cell line in Figure 3.6A). To determine whether these signals represented genuine epitope-specific cells or unspecific staining, an attempt was made to selectively induce epitope-specific CD8 T cell proliferation by stimulation of the cultures with *T. annulata*-infected cells pulsed with the respective peptides. However, as shown in Figure 3.9, there was no apparent expansion of $Tp2_{98\text{-}106}$ tetramer-positive CD8 T cells after two rounds of peptide stimulation. Instead, the percentage of positive signals decreased from 2% before peptide stimulation to 0.5% after peptide stimulation.

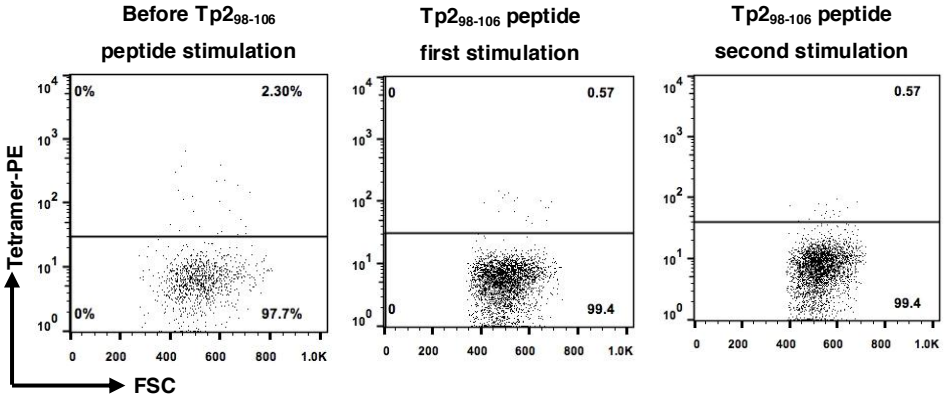


Figure 3.9 Stimulation of CD8 T cells with autologous *T. annulata* cells pulsed with peptide.
Tetramer staining showed no expansion of detectable $Tp2_{98\text{-}106}$-specific CD8 T cells was evident in a CD8 T cell lines from animal 702197 after either 1 or 2 stimulations with $Tp2_{98\text{-}106}$ peptide.

Magnetic beads-based cell sorting (MACS) of pMHC I tetramer stained populations has been used successfully to detect and quantify low frequency antigen-specific CD8 T cells from naïve mice [Obar et al., 2008]. As an alternative approach to determine if very low frequency tetramer positive CD8 T cells were present in the cultures derived from the 3 animals which had been considered to be Tp2 negative, a similar technique but employing flow cytometric cell sorting rather than MACS was used. Representative data from this analysis (from animal 402145) is presented in Figure 3.10. 1000 (0.1%) $CD8^{+}Tp2_{98\text{-}106}{}^{+}$, 3000 (0.3%) $CD8^{+}Tp2_{49\text{-}59}{}^{+}$ and 100 (0.01%) $CD8^{+}Tp2_{50\text{-}59}{}^{+}$ cells (cells in red quadrants) were sorted from 10^6 cultured CD8 T cells, re-stimulated with autologous TpM and then tetramer staining performed 7 days later. As shown in Figure 3.10A, a clear positive population (10%) specific for the $Tp2_{98\text{-}106}$ epitope was detected for animal 402145, whereas neither clear $Tp2_{49\text{-}59}$ nor $Tp2_{50\text{-}59}$-specific responses were evident. From the above results, the percentage of $Tp2_{98\text{-}106}$ epitope-specific cells in the CD8 T cell enriched cell line from animal 402145 was estimated to be approximately 0.01% (0.1% sorted cells × 10% $tetramer^{+}$ cells). Identical analyses performed for animals 702162 (A10/N5) and 702197 (A10/A11) failed to identify $Tp2_{98\text{-}106}$, $Tp2_{49\text{-}59}$ or $Tp2_{50\text{-}59}$-specific populations (data not shown).

Together, these results suggested that i) an additional A10-heterozygous animal had a $Tp2_{98\text{-}106}$ epitope-specific CD8 T cell (although this was veryminor) but no $Tp2_{49\text{-}59}$ or $Tp2_{50\text{-}59}$-specific populations could be identified in any of the A10-heterozygous animals analysed; and ii) using the FACS-enrichment technique, reliable detection of pMHC I tetramer positive CD8 T cells could be as low as 1 in 10^5.

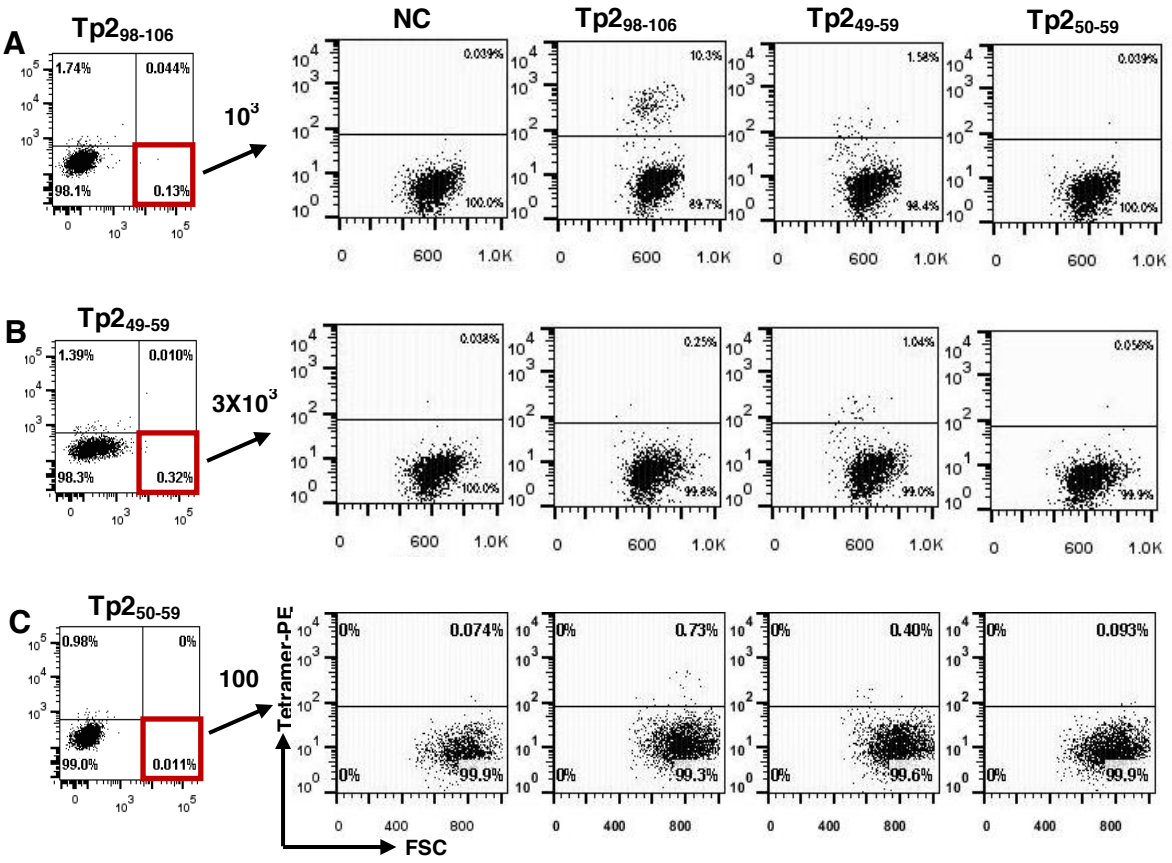


Figure 3.10 *In vitro* enrichment and expansion of $Tp2_{98-106}$-specific CD8 T cells from A10 heterozygous animal 402145.

A CD8 T cell line from animal 402145 was stained with tetramers specific for the three defined Tp2 epitopes (A, $Tp2_{98-106}$; B, $Tp2_{49-59}$; C, $Tp2_{50-59}$) and antibodies specific for CD4, γδ T cells and NK cells. Cells in the red quadrants represent CD8 T cells positive for tetramers. After sorting and *in vitro* expansion, these CD8+tetramer+ cells were stained with the three defined tetramers to detect epitope-specific CD8 T cells.

3.4 Discussion

This chapter reports the results of quantitative analyses of CD8 T cells specific for the three defined BoLA 2*01201-restricted epitopes within the Tp2 antigen of *T. parva* in a series of A10-homozygous and -heterozygous animals. Previous work had been largely restricted to analysis of *in vitro* established CD8 T cell lines from two immunized A10-homozygous cattle [MacHugh et al., 2009], using recognition of peptide-pulsed targets to determine epitope-specificity. Such approaches due to their laborious nature limit the number of lines that can be effectively analysed and also have constraints with regard to the accuracy of defining the genuine epitopes (Connelley et al. – in preparation). By using pMHC I tetramers to analyse the frequency of epitope-specific responses in this current work it has been possible to extend the studies of the Tp2-specific responses to a larger number of animals, to *in vitro* cell lines generated under a variety of conditions and also to direct *ex vivo* quantification – thereby refining our understanding of the relative dominance of the 3 epitopes and the factors that may influence this.

CD8 T cell responses against the $Tp2_{49\text{-}59}$ epitope showed consistent dominance in all 5 BoLA-A10 homozygous animals examined. However, the epitope specificity profile of responses in the A10-heterozygous animals differed in two respects from that of the homozygous animals. First, only 4 of the 6 heterozygous animals had a detectable Tp2 antigen-specific CD8 T cell response, and in one of these the specific T cells were at very low frequencies. Second, in those heterozygous animals that had a detectable response, the specific CD8 T cells were focused solely on the $Tp2_{98\text{-}106}$ epitope, with no detectable CD8 T cells specific for the $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitopes. These observations indicate that the presence of the MHC class I allele 2*01201 alone does not predict the Tp2 epitope specificity of the CD8 T cell response in A10+ animals and that the presence of other expressed MHC alleles in heterozygous animals strongly influences the epitope specificity of the response to this antigen. The factors that might have affected these findings, including the potential influence of *in vitro* manipulation of the CD8 T cell cultures and use of different stimulator cells for antigen presentation, were explored in a further series of experiments.

The 6 A10-heterozygous animals examined consisted of 3 pairs that had identical class I MHC haplotypes. The initial failure to detect Tp2-specific CD8 T cells in one animal of each pair suggested that the parasite-infected cells used as stimulators to generate the T cell cultures from these animals might be defective in their ability to stimulate Tp2-specific CD8 T cells *in vitro*. In other systems, intrinsic differences of antigen presenting cells, such as factors related to antigen digestion, transport and peptide trimming, have been reported to influence immunodominance of CD8 T cell responses in other systems [Tenzer et al., 2009]. However in the current study, CD8 T cell cultures derived from each animal by stimulation with either autologous or MHC identical infected cells exhibited similar specificities, indicating that the difference between the animals was not due to a defect in the stimulator cells.

Well-established culture methods were applied [Goddeeris and Morrison, 1988] for *in vitro* expansion of memory CD8 T cells from the immune cattle. Although such methods have provided valuable data, results from studies of other models using similar methodology [Koning et al., 2014, Belyakov et al., 2001] have indicated that changes in the clonotypic composition of CD8 T cells can occur after repeated *in vitro* stimulation, especially for T cell populations with high clonotypic diversity. Although the basis of the changes were not determined, it was proposed that *in vitro* manipulation could cause selective expansion of T cell clonotypes with high avidity, which if pronounced could result in the alteration of epitope specificity of the cultured CD8 T cell population [Price et al., 2005].

In the current study, this was investigated by examining the specificity of cultures generated following a single *in vitro* stimulation of purified CD8 T cells with irradiated cells. The epitope specificities of these primary cultured CD8 T cell lines derived from both A10-homozygous and heterozygous animals generally showed the same profiles of epitope dominance hierarchy as the cultures obtained following 3 *in vitro* stimulations, although the percentages of epitope-specific CD8 T cells were generally lower in the former, reflecting more limited expansion of the epitope-specific CD8 T cells from the memory T cell pools. An interesting observation was the apparent different kinetics of expansion of the CD8 T cells

specific for the $Tp2_{50-59}$ and $Tp2_{49-59}$ epitopes during primary culture of CD8 T cells from one of the A10-homozygous animals (animal 403957, Figure 3.4C). The response to $Tp2_{50-59}$ was initially dominant and was followed by a drop of percentage of these cells on day 13 of cell culture when the $Tp2_{49-59}$ epitope became dominant. Variation in a number of parameters, including precursor frequency of the specific T cells, epitope abundance on the infected cells used for stimulation and the affinities of the TCRs of the responding T cells for the peptide-MHC I complex, could account for these different expansion rates of epitope-specific CD8 T cells. In spite of these observations, the $Tp2_{49-59}$ epitope dominance in A10-homozygous cattle and the $Tp2_{98-106}$ epitope-dominance in A10-heterozygous cattle were confirmed in the primary CD8 T cell cultures.

Although responses to the $Tp2_{49-59}$ epitope predominated in all five A10 homozygous cattle, the percentages of $Tp2_{50-59}$-specific CD8 T cells were higher in two of the animals (592 and 1011) compared to the other three cattle. Previous studies of CD8 T cell clones from these two cattle had shown that over 70% of CD8 T cell clones recognised the $Tp2_{49-59}$ peptide [MacHugh et al., 2009]. At that time, the existence of two distinct epitopes within the $Tp2_{49-59}$ sequence had not been recognised. The CD8 T cell lines from these animals examined in the current study had been subjected to an additional 2-3 *in vitro* passages. It is therefore possible that this resulted enrichment of the $Tp2_{50-59}$-specific populations in these cultures, although in the absence of comparative data from earlier passages, this remains speculative.

The complete absence of Tp2-specific CD8 T cell responses in some animals might be due to the response being masked by a dominant response restricted by the non-A10 MHC haplotype. Moreover, the absence of detectable responses to the $Tp2_{50-59}$ and $Tp2_{49-59}$ epitopes could potentially be due to more efficient presentation of these epitopes by A10-homozygous infected cells compared to heterozygous cells. It has been reported that diversified MHC class I genotypes in heterozygous individuals could increase the competition between different alleles for surface expression and result in altered epitope presentation for CD8 T cell activation and proliferation [Tourdot and Gould, 2002]. These possibilities were investigated by

examining the responses elicited by stimulating CD8 T cells from the A10-heterozygous animals with A10-homozygous stimulator cells. However, CD8 T cells specific for $Tp2_{49-59}$ and $Tp2_{50-59}$ were still not detected in these cultures; nor did stimulation with A10-homozygous cells reveal $Tp2_{98-106}$-epitope-specific CD8 T cell responses in animals that originally did not have any detectable Tp2-specific response. A 1.5-3 fold increase in the proportions of $Tp2_{98-106}$-specific CD8 T cells was observed in the cultures from the three Tp2-positive A10-heterozygous animals, compared to cultures generated from the same animals with autologous A10-heterozygous stimulator cells. This finding suggested that these animals may have generated CD8 T cell responses restricted by products of the non-A10 MHC I haplotype, which would not have been present in CD8 T cell cultures generated with A10-homozygous stimulator cells. This point will need to be clarified in further experiments.

Further evidence that the Tp2 epitope specificities detected in CD8 T cell lines are representative of the responses of these BoLA-A10 cattle was obtained by examining the *in vivo* response in 4 of the cattle following parasite challenge. Tetramer staining again demonstrated a dominant response to the $Tp2_{49-59}$ epitope in A10-homozygous cattle and the $Tp2_{98-106}$ epitope in A10-heterozygous cattle. For homozygous cattle, the percentage of $Tp2_{49-59}$-specific CD8 T cells was much lower in one of the A10-homozygous animal (403992 - 0.5%) than the other animal (302186 - 7.5%). This difference might relate to earlier clearance of the challenge infection in this animal and hence a less pronounced expansion of the specific memory CD8 T cells. Nevertheless, these results suggest that, in terms of epitope dominance hierarchy, the composition of *in vitro* CD8 T cell lines are representative of *in vivo* antigen-specific CD8 T cell responses against *T. parva*.

Staining of T cells with fluorochrome-conjugated pMHC I tetramers is the most direct means of identifying epitope-specific CD8 T cells and has been used extensively to quantify CD8 T cell responses. In comparison with other methods, which in general rely on detection of T cell function such as cytokine secretion or cytotoxicity, tetramers are more sensitive for enumerating T cells responding to relatively dominant antigens. However, their main disadvantage is lack of sensitivity

in detecting T cells present at low frequency. This is because of the limitations of flow cytometry in distinguishing small numbers of genuinely positive signals from non-specific background staining. Various approaches have been taken to improve accurate detection of low-frequency antigen-specific CD8 T cells by tetramers [Wooldridge et al., 2009, Chattopadhyay et al., 2008]. Magnetic-beads-based enrichment of tetramer-positive cells prior to performing flow cytometry has been used to quantify rare positive cells from naïve T cell populations [Obar et al., 2008, Moon et al., 2007]. An attempt was made to use a two-step approach in the current study to detect cells specific for all 3 Tp2 epitopes in the three A10-heterozygous cattle that did not show any detectable Tp2-specific CD8 T cell responses. Cell sorting was initially used to collect rare positive events and the resultant cells subjected to antigenic stimulation in culture. In one of the 3 animals, 10% $Tp2_{98\text{-}106}$ tetramer-positive cells were detected in the CD8 T cell culture established by sorting positive events present at 0.1% using the respective tetramer. This would indicate that the original CD8 T cell population contained approximately 0.01% epitope-specific T cells. This is in line with the reported lowest level of antigen-specific CD8 T cells detectable by tetramer staining [Singh et al., 2013, Comin-Anduix et al., 2006]. The absence of detectable tetramer-positive cells in cultures of the other sorted populations indicates that there was either no response or aminimal response to the epitopes in these animals.

The data presented in this chapter clearly demonstrate that the dominance hierarchies of the 3 Tp2 CD8 T cell epitopes differs between A10-homozygous and A10-heterozygous animals. The *ex vivo* results demonstrated that this was not due to an artefact of the *in vitro* culture systems and indicated that the specificities of the CD8 T cell cultures reflected the *in vivo* CD8 T cell responses. While all A10-homozygous animals generated CD8 T cell responses to all 3 Tp2 epitopes, the responses in A10-heterozygous animals were variable and when present only recognised one of the 3 Tp2 epitopes. When compared with previous observations in an A10/A18 heterozygous cattle, where CD8 T cell responses to the $Tp2_{49\text{-}59}$ epitope were detected using ELISPOT assay [Graham et al., 2008], results in the current study suggests that discrepancies of MHC I genotypes and detection approaches may

induce the different profiles of Tp2 antigen specificities in A10-heterozygous cattle. Further investigation is required to explore the reasons underlying the absence of any responses to $Tp2_{49-59}$ and $Tp2_{50-59}$ epitopes in the A10-heterozygous animals. Two possibilities are proposed. First, the MHC-heterozygosity may in some way alter the processing of these epitopes thus altering their density on the surface of the infected cells. Second, expression of other MHC I alleles may result in negative selection of the TCRs specific for $Tp2_{49-59}$ and $Tp2_{50-59}$ thus reducing the repertoire of T cells capable of responding to these epitopes.

The fact that the three epitopes are derived from the same antigen and presented by the same MHC I allele provides a model system to investigate the potential influence of T cell repertoires to epitope specificity of CD8 T cell responses. Therefore, following work were conducted to initially analyse clonotype compositions of Tp2 epitope-specific CD8 T cells from both A10-homozygous and –heterozygous cattle.

Chapter 4: TRB repertoire analyses for Tp2 epitope-specific CD8 T cell responses

4.1 Introduction

Antigen specificity of CD8 T cells is determined by the T cell receptor (TCR), a cell surface heterodimer composed of α and β chains. During T cell development, TCR diversity is generated through recombination of variable (V), diversity (D) and joining (J) gene segments for the β chain and V and J gene segments for the α chain. Nucleotide additions and deletions at the junctions between V(D)J produce the complementarity determining region 3 (CDR3). Together with CDR1 and CDR2 regions, encoded within the variable gene of each chain, they form the six loops of the αβTCR that interact with peptide-MHC I (pMHC I) complexes presented on the surface of antigen presenting cells (APCs) or infected cells [Garboczi et al., 1996, Market and Papavasiliou, 2003, Stewart-Jones et al., 2003]. Somatic recombination, which occurs in developing T cells within the thymus, draws on multiple germline-encoded V, D and J gene segments. Different combinations of these gene segments, together with junctional diversity, have been estimated in humans to have the potential to generate between 10^{15} and 10^{20} unique αβTCRs [Lieber, 1991, Davis and Bjorkman, 1988]. However, many combinations are non-functional or are deleted during development because of their ability to recognise self-peptide-MHC. A direct estimate showed that naïve human peripheral blood contains at least ~10^{7} αβTCRs at any given time [Arstila et al., 1999]. The size of the αβTCR repertoire for naïve mouse T splenocytes was also estimated to be ~10^{6} unique αβTCRs [Casrouge et al., 2000].

The highly diverse TCR repertoire is believed to be of benefit by enabling the adaptive immune system to recognize a large variety of pathogen-derived peptides [Nikolich-Zugich et al., 2004]. In general, the T cell response specific for a given peptide-MHC includes T cells with different TCR rearrangements utilising different gene segments. The benefits, if any, of this diversity in generating effective pathogen-specific immune protection are still unclear. One proposal is that generation of heterogeneous CD8 T cell clonotypes with multiple choices of

structural and functional avidity for immunodominant epitope recognition collectively enhances the efficacy of the immune response andminimises the likelihood of mutational escape from immune control [Belz et al., 2001, Charini et al., 2001]. Evidence for such an effect was reported in CD8 T cell responses against an immunodominant Herpes Simplex Virus (HSV) glycoprotein B-derived peptide (495-502; SSIEFARL). Animal survival observed in H-2K^{bm8} mice was associated with a diverse epitope-specific CD8 T cell repertoire, while mice with a less diverse K^{b}-specific repertoire showed death after infection [Messaoudi et al., 2002]. In this case, the protection provided by diverse TCR repertoire was illustrated to be associated with a greater range of high avidity TCRs, which were demonstrated to be more effective than low avidity TCRs against pathogen infection [Derby et al., 2001]. For pathogens with rapid evolving rate, such as primate immunodeficiency viruses, TCR diversity has been suggested to potentially recognize an increased spectrum of epitope mutants allowing more effective control of mutated viruses [Douek et al., 2002, Price et al., 2004].

Despite the advantages of TCR diversity, TCR β-chain (TRB) analyses of epitope-specific CD8 T cells in several virus disease models have shown substantial variation in repertoire usage. For example, CD8 T cells specific for a subdominant epitope in Epstein-Barr virus (EBV) nuclear antigen 4 (EBNA4 399-408) showed highly restricted Vβ usage, while CD8 T cells specific for a dominant epitope in the same antigen (EBNA4 416-424) had a broad repertoire usage [Campos-Lima et al., 1997]. The TCR diversity was proposed to influence the immunodominance hierarchy of CD8 T cell responses. A correlation of TCR diversity with magnitude of the CD8 T cell response has also been reported in individuals with HIV infection [Balamurugan et al., 2010]. However, such a correlation was not significant for other virus disease models [La Gruta et al., 2008, Koning et al., 2013].

Epitope dominance hierarchy has potential implications for vaccine development. The results of the previous chapter demonstrated dominance hierarchies of three defined *T. parva* epitopes from the same parasite protein (Tp2) presented by the same MHC class I allele (BoLA 2*01201), which differed between A10-homozygous and -heterozygous cattle. This chapter aims to explore potential

mechanistic explanations for this phenomenon, by examining the TCR repertoires of the responding CD8 T cells.

Previously, TRB repertoires of Tp2 epitope-specific CD8 T cells were analysed using a method combining CD8 T cell cloning and Vβ subfamily-specific PCR and sequencing [Connelley et al., 2011, Connelley et al., 2008]. However, the generation of T cell clones is extremely time-consuming. Therefore, the current study set out to use a template-switch anchored RT-PCR, for direct analysis of purified epitope-specific T cell populations. By using a forward primer designed according to the anchor sequence added to the 5'-terminus of full-length mRNA during reverse-transcription and a reverse primer specific for the constant region of bovine TRB genes, the rearrangement of bovine TRB can be amplified without bias in composition [Quigley et al., 2011, Douek et al., 2002]. The generation of pMHC I tetramers incorporating the defined Tp2 epitopes facilitated purification of epitope-specific CD8 T cells from both *in vitro* and *ex vivo* T cell populations. Direct analysis of T cells harvested *ex vivo* has the advantage of avoiding potential bias as a result of outgrowth of some clonotypes during *in vitro* expansion [Dietrich et al., 1997, Davis et al., 2011].

4.2 Materials and methods

4.2.1 Enrichment of epitope-specific CD8 T cells

Tetramer staining combined with magnetic beads based cell sorting (MACS) were used for enrichment of epitope-specific CD8 T cells from PBMC isolated from immune cattle at the peak of the active CD8 T cell response following parasite challenge. Briefly, 1×10^8 PBMC were stained with PE-labelled pMHC I tetramer for 30min at 4°C in MACS buffer (PBS containing 0.5% BSA and 2mM EDTA). Cells were then washed and labelled with anti-PE antibody coated microbeads (Miltenyi Biotec) for 30min at 4°C, washed and passed through a magnetized LS column (Miltenyi Biotec). Columns were then washed and removed from the magnet, and bound cells were eluted with standard cell culture medium. Cells were then stained with an antibody cocktail reactive with all lineages except CD8 T cells (as described in section 3.2.6) for identification of tetramer positive CD8 T cells. Sorted cells were examined using a FACSCalibur cell analyser and if necessary to obtain sufficient purity (>98%), tetramer positive CD8 T cells were sorted using a FACSAria III.

4.2.2 Template switch anchored RT-PCR

Details of TRB repertoire analysis using the template switch anchored RT-PCR were described in section 2.3.1. Briefly, mRNA was isolated from purified epitope-specific CD8 T cells and used for cDNA synthesis. To obtain full-length cDNA, a template-switch reverse transcription method was used employing the two intrinsic properties of Moloney murine leukemia virus (MMLV) reverse transcriptase – reverse transcription and template switching [Zhu et al., 2001]. Because the template switching activity ofmMLV reverse transcriptase only happens when the enzyme reaches the end of RNA, full-length cDNA can be enriched and prematurely terminated cDNA are eliminated. An anchor sequence is added to the 5'-end of mRNA during the process of reverse transcription. Primers specific for this anchor sequence and the constant region of the bovine TRB gene were used for PCR amplification of TRB transcripts. PCR products were purified and subcloned for Sanger sequencing.

4.2.3 PCR amplification of TRB transcripts with defined Vβ and Jβ gene segments

Specific primers were designed to amplify one particular VDJ rearrangement incorporating the Vβ28 and Jβ3.2 gene segments. Forward and reverse primers used were VB28F 5'-CGCCAAGATCCGGGATTTG-3' and JB3.2R 5'-CAGCACAGTCAGCTTGGAAC-3'. The PCR protocol used was performed as described previously [Connelley et al., 2008]. Briefly, total RNA was extracted from target samples using Tri-reagent (Sigma-Aldrich, Poole, Dorset, UK) and cDNA subsequently synthesised using the Reverse Transcription System (Promega, Madison, WI, USA) with priming by the Oligo (dT)15 primer, according to the manufacturers' instructions. A single-round PCR was conducted in reactions composed of 1 µ cDNA template, 10 pmol each of the forward and reverse primers, 0.5 units of Biotaq (Bioline, London, UK) and 2µl SM-0005 10× buffer (ABgene, Epsom, Surrey, UK) per 20µl reaction. Cycling conditions were 5min at 94 ℃, 5 cycles of 1min at 94 ℃, 1min at 60 ℃, 1min at 72 ℃), 25 cycles of 30 s at 94 ℃, 1min at 60 ℃, 1min at 72 ℃ and a final extension period of 5min at 72 ℃. PCR products were analysed in 1.5% agarose gels and visualised using the G:Box system (Syngene, UK) after staining with GelRed Nucleic Acid Gel Stain (Biotium, Inc. Hayward, CA).

4.2.4 Analysis of sequence data

Sequences of TRB transcripts obtained from epitope-specific CD8 T cells (Sanger sequencing) were analyzed according to the method described in section 2.3.2.

4.3 Results

4.3.1 Development of the template-switch anchored RT-PCR for TRB repertoire analysis

Initial attempts to use a published protocol [Quigley et al., 2011] for obtaining TRB transcripts from sorted epitope-specific CD8 T cells from A10 immune cattle were unsuccessful. Successful amplification of TRB transcripts from unsorted cultured CD8 T cells using the same process suggested that either (1) cell staining and sorting was causing a deterioration of yield and/or quality of mRNA or (2) inadequate numbers of sorted cells were collected for downstream work. A variety of modifications to the protocols for mRNA isolation and cDNA synthesis from sorted cells were attempted without success.

To circumvent this technical problem and obtain sufficient numbers of epitope-specific CD8 T cells with high purity and retain high quality mRNA, tetramer positive cells from primary CD8 T cell cultures (day 9 for A10-homozygous and day 11 for A10-heterozygous) were first sorted into 10-20 wells of a 96-well plate at 10^2-10^3 cells per well and stimulated with autologous parasitized cells in the presence of rIL2 to amplify the cell numbers, as shown in Figure 4.1. After 7 days in culture the expanded cells (with a starting number of 1000 - 20,000 sorted pMHC I tetramer positive cells) were collected and checked for epitope specificity and purity, and cultures containing ≥98% positive cells were used directly for TRB repertoire analysis. Cultures with less than 98% purity were re-sorted to enhance cell purity - PCR amplification from these sorted cells was successful (suggesting that if the starting population was sufficiently enriched to subject the cells to only aminimal sorting time, the quality and quantity of mRNA was adequate for subsequent cDNA synthesis). The resultant cells were then stored into RNAlater for mRNA isolation and TRB repertoire analysis according to the method described in section 2.3.1.

Sequence data were generated by subcloning the RACE-PCR amplicons and Sanger sequencing. For each purified epitope-specific CD8 T cell population, more than 50 recombined in-frame sequences with defined V, J and CDR3 regions were obtained, as shown in Table 4-1.

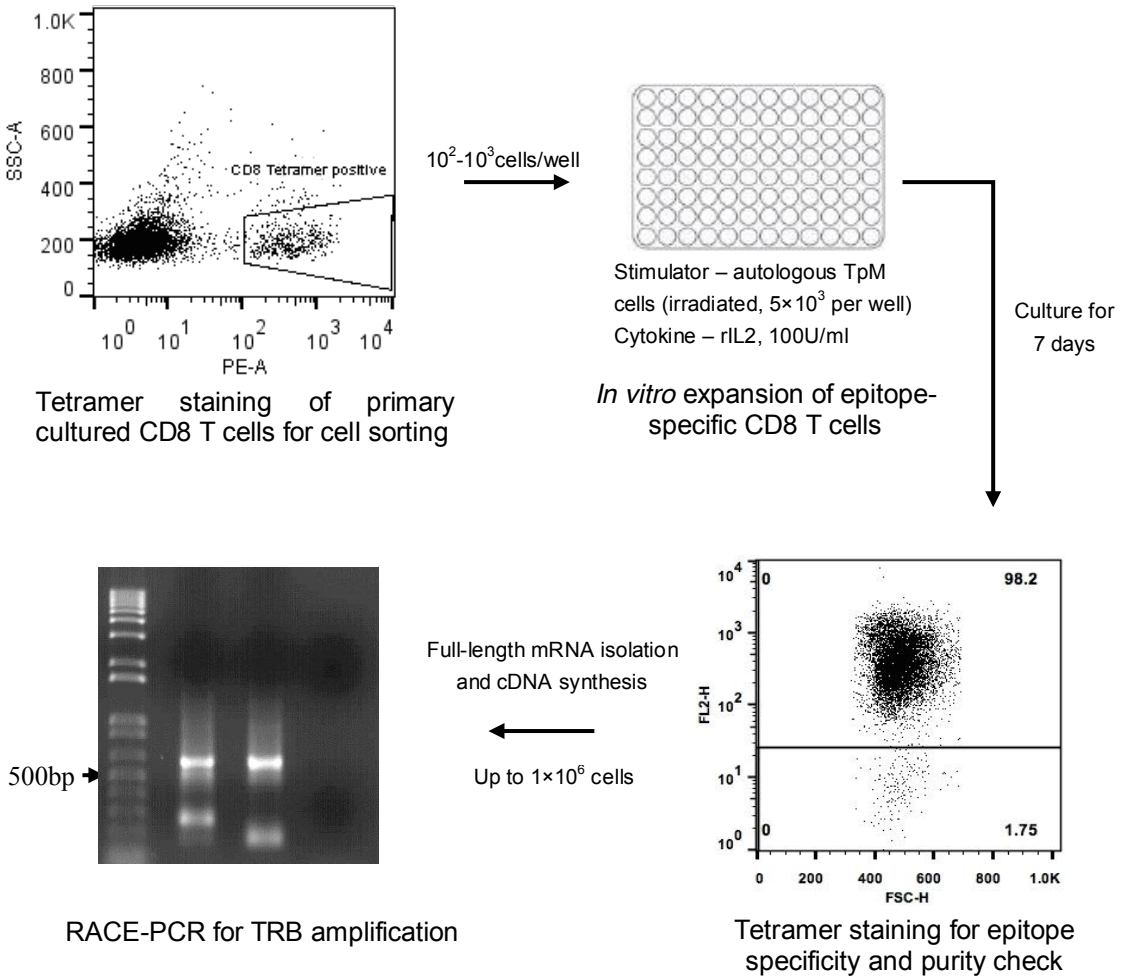


Figure 4.1 Illustration of preparing epitope-specific CD8 T cells for TRB repertoire analysis.
Epitope-specific CD8 T cells from primary cultured CD8 T cell lines were sorted and cultured for 7 days with the presence of stimulators (irradiated autologous TpM) and rIL2 (100U/ml). Epitope specificity and purity (≥98%) were determined prior to mRNA isolation. Using the established template-switch anchored RT-PCR, TRB transcripts of epitope-specific CD8 T cells were amplified with the expected DNA size of between 500-700bp.

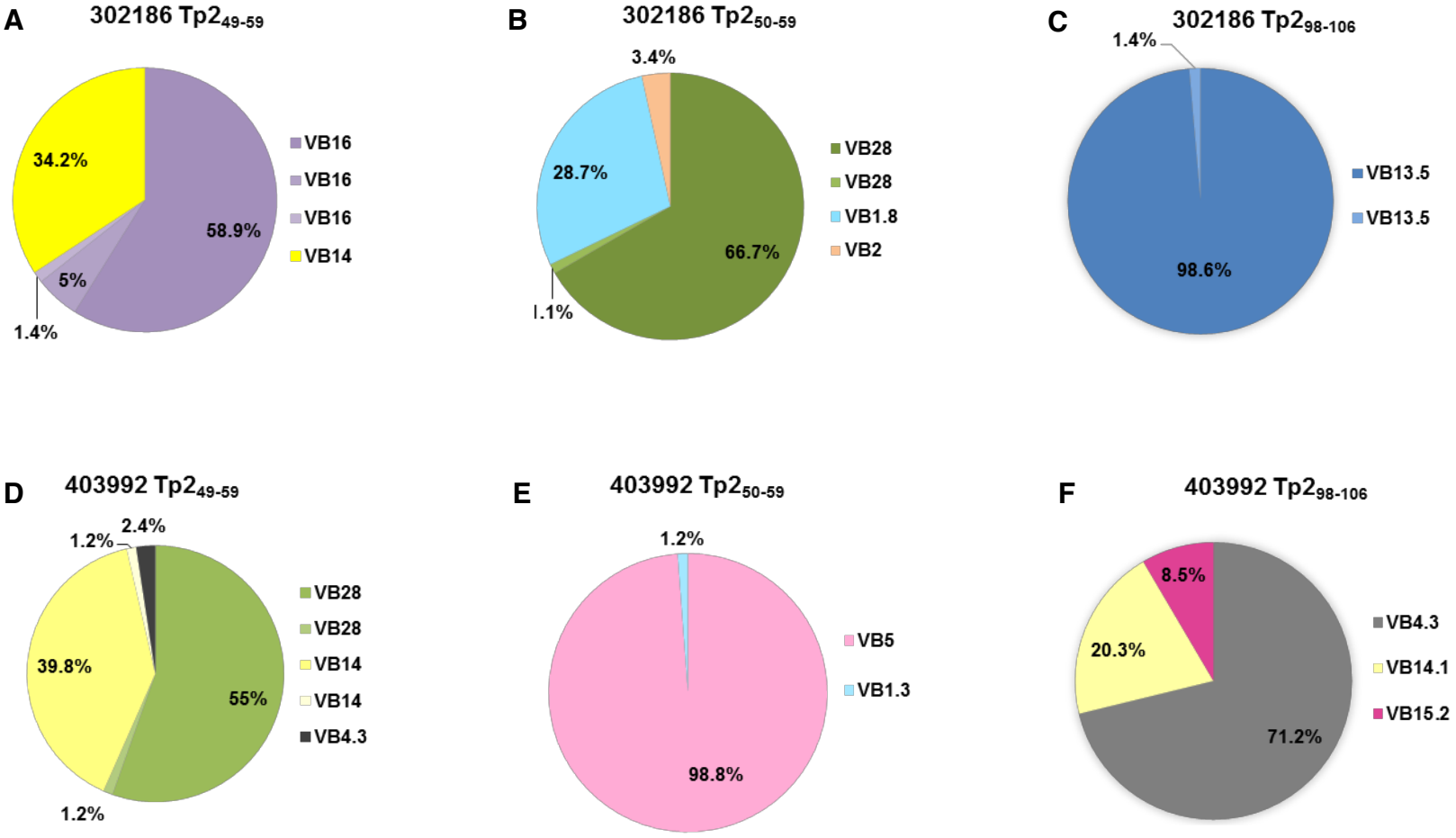
Table 4-1 Total number of in-frame TRB sequences for epitope-specific CD8 T cells obtained from both A10-homozygous and A10-heterozygous cattle

MHC I haplotype	Animal	$Tp2_{49-59}$	$Tp2_{50-59}$	$Tp2_{98-106}$
A10/A10	**302186**	73	87	73
A10/A10	**403992**	83	84	59
A10/A10	**403957**	52	63	81
A10/A12	**102121**			74
A10/N5	**102170**			86
A10/A11	**402082**			85

The numbers of sequencing reads with in-frame TRB chain sequences are shown for 9 Tp2 epitope-specific CD8 T cell populations derived from 3 A10-homozygous cattle and for 3 Tp2 epitope-specific CD8 T cell populations derived from 3 A10-heterozygous cattle.

4.3.2 TRB clonotype compositions for Tp2 epitope-specific CD8 T cells from A10-homozygous cattle

The epitope dominance hierarchy of CD8 T cell responses specific for the three defined Tp2 epitopes - $Tp2_{49-59}$, $Tp2_{50-59}$ and $Tp2_{98-106}$ - has been verified for A10-homozygous cattle, showing a highly dominant response to the $Tp2_{49-59}$ epitope and smaller responses to the $Tp2_{50-59}$ and $Tp2_{98-106}$ epitopes (see section 3.3.1). T cell receptor β chain repertoires for CD8 T cells specific for the three epitopes were initially analysed for the three A10-homozygous cattle. The TRB clonotype composition for each of the purified epitope-specific CD8 T cell populations are shown in Figure 4.2. The results identified a limited number of expanded TRB clonotypes in the CD8 T cells specific for both the dominant $Tp2_{49-59}$ epitope and the subdominant $Tp2_{50-59}$ and $Tp2_{98-106}$ epitopes. Clonal expansion of one or two dominant TRB clonotypes was observed for responses to each epitope in each of the animals.


A
302186 Tp2$_{49-59}$
34.2%
58.9%
5%
1.4%
VB16
VB16
VB16
VB14
B
302186 Tp2$_{50-59}$
3.4%
28.7%
66.7%
1.1%
VB28
VB28
VB1.8
VB2
C
302186 Tp2$_{98-106}$
1.4%
98.6%
VB13.5
VB13.5
D
403992 Tp2$_{49-59}$
1.2%
2.4%
39.8%
55%
1.2%
VB28
VB28
VB14
VB14
VB4.3
E
403992 Tp2$_{50-59}$
1.2%
98.8%
VB5
VB1.3
F
403992 Tp2$_{98-106}$
8.5%
20.3%
71.2%
VB4.3
VB14.1
VB15.2

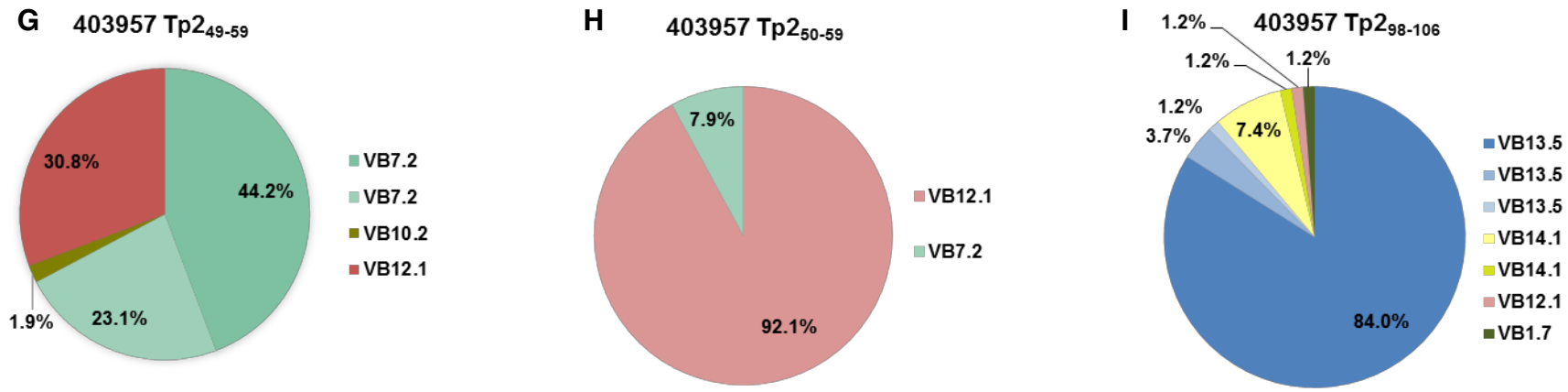


Figure 4.2 TRB clonotype distributions for Tp2 epitope-specific CD8 T cell populations obtained from three A10-homozygous cattle. A, D and G, $Tp2_{49-59}$ epitope-specific TRB clonotype compositions for animal 302186, 403992 and 403957 respectively; B, E and H, $Tp2_{50-59}$ epitope-specific TRB clonotype compositions for animal 302186, 403992 and 403957 respectively; C, F and I, $Tp2_{98-106}$ epitope-specific TRB clonotype compositions for animal 302186, 403992 and 403957 respectively. Each clonotype is represented by a unique colour. Vβ gene segments for identified clonotypes are shown. Clonotypes with the same Vβ gene but different CDR3β and/or Jβ genes are shown in different colours. Percentage of nucleotide sequence reads for each clonotype is illustrated.

The results of analyses of the sequences of all identified TRB clonotypes, including the V and J gene usage and the sequences of the CDR3s, are shown in Table 4-2. Some low-abundant clonotypes had the same Vβ and Jβ gene segments as a highly dominant clonotype, but had one or two amino acid differences within the translated CDR3β sequence. To investigate the possibility of sequencing errors, the sequences were examined to determine whether the substitutions were located within parts of the CDR3β sequence derived from germline V, D and J genes. Three amino acid substitutions (in red font) appeared within germline J segments of the CDR3β region. Therefore, these were considered to have arisen from PCR or sequencing errors, since germline TCR gene segments do not undergo somatic mutation [Bolotin et al., 2012]; and accordingly the sequences were corrected for subsequent analyses. Eight amino acid substitutions (in purple font and underlined) within junctions could have been generated by nucleotide additions or deletions during TCR rearrangement and although it is also possible that they were due to PCR errors; these were not corrected. Two examples of clonotypes incorporating the same Vβ and Jβ gene segments present in both the $Tp2_{49-59}$ and $Tp2_{50-59}$ epitope-specific CD8 T cells were observed (shown in green and brown font). For one of these pairs (brown), the CDR3 sequences differed in both lengths and amino acid sequences and clearly represented different clonotypes. However, the other clonotype pair (shown in green font), both from animal 403957, had identical CDR3β sequences. This clonotype is dominant (44.2%, 23 of the 52 sequences reads) in $Tp2_{49-59}$ epitope-specific CD8 T cell population of this animal, and represented 7.9% of sequences (5 of the 63 sequence reads) obtained from the $Tp2_{50-59}$ epitope-specific CD8 T cells. The numbers of reads in the latter population seems too high to be due to contamination of tetramer-negative cells in the sorted population. It is therefore possible that the $Tp2_{50-59}$ tetramer cross-reacts to some degree with the $Tp2_{49-59}$ epitope-specific T cells expressing this particular TRB clonotype.

The numbers of unique TRB clonotypes for each epitope-specific CD8 T cell population, following corrections referred to above, are shown in Figure 4.3. Very limited TRB clonotype diversity was observed within the 52-86 sequences obtained from T cells specific for each the of the three Tp2 epitopes in each individual animal,

although one $Tp2_{98-106}$-specific CD8 T cell population from animal 403957 showed more clonotypes than the other populations. The total numbers of clonotypes obtained from the three A10-homozygous cattle are 11 for the dominant $Tp2_{49-59}$ epitope, 8 for the $Tp2_{50-59}$ epitope and 13 for the $Tp2_{98-106}$ epitope. Sharing of gene segments was observed between different epitope-specific clonotypes; however, different CDR3β sequences for these clonotypes suggest their unique specificity.

Table 4-2 Sequences of Tp2 epitope-specific TRB repertoires from A10 homozygous cattle

Epitope	Sequence					Number	Freq (%)	Animal
	Vβ	FR	CDR3β	FR	Jβ			
$Tp2_{49-59}$	14	CAS	SRYAGPETL	YFG	2.4	25	34.2	302186
	16	CAS	SLQPFYDY	HFG	1.2	43	58.9	302186
	16	CAS	SLQPFYVY	HFG	1.2	4	5.5	302186
	16	CAS	SLQPFYGY	HFG	1.2	1	1.4	302186
	4.3	CSA	TRGGREDY	HFG	1.2	2	2.4	403992
	14	CAS	SEWNTNGPL	YFG	3.2	33	39.8	403992
	14	CAS	SGWNTNGPL	YFG	3.2	1	1.2	403992
	28	CAT	AADLDDNPL	YFG	3.3	46	55.4	403992
	28	CAT	AADLDDDPL	YFG	3.3	1	1.2	403992
	7.2	CAS	SRDLVAETL	YFG	2.4	23	44.2	403957
	7.2	CAS	STVTAQIQ	YFG	3.5	12	23.1	403957
	10.2	CAS	SQAEVSGGAPWIPL	YFG	3.3	1	1.9	403957
	12.1	CAS	SYGGSGSYEQ	YFG	3.7	16	30.8	403957
$Tp2_{50-59}$	1.8	CAS	SQDPETL	YFG	2.4	25	28.7	302186
	2.7	CGA	RGLGEV	FFG	1.1	3	3.4	302186
	28	***CAS***	***AEYGGENTQPL***	***YFG***	***3.2***	***58***	***66.7***	***302186***
	28	CAS	AEYGGKNTQPL	YFG	3.2	1	1.1	302186
	1.3	CAS	SPLGGYRSAVQL	YFG	2.2	1	1.2	403992
	5	CAS	VSPGGDY	HFG	1.2	83	98.8	403992
	7.2	CAS	SRDLVAETL	YFG	2.4	5	7.9	403957
	12.1	CAQ	HSSREQ	YFG	3.7	58	92.1	403957

(Table continues)

(Continued Table)

Epitope	Sequence					Number	Freq (%)	Animal
	Vβ	FR	CDR3β	FR	Jβ			
$Tp2_{98-106}$	13.5	CTS	NLGGLDLGTQ	YFG	2.3	72	98.6	302186
	13.5	CTS	TFGGWALETL	YFG	2.4	1	1.4	302186
	4.3	CSA	GSGYEQ	YFG	3.7	42	71.2	403992
	14	CAS	SPGLGVQIQ	YFG	3.5	12	20.3	403992
	15.2	CAS	NAGQQGGTQPL	YFG	3.2	5	8.5	403992
	1	CAS	SVRGGDTQ	YFG	2.3	1	1.2	403957
	1.7	CAS	SQDYGATL	YFG	2.4	1	1.2	403957
	12.1	CAT	LILAREQ	YFG	3.7	1	1.2	403957
	13.5	CTS	SRGGRIDGEL	HFG	3.1	3	3.7	403957
	13.5	CTS	SPGGGRIDGEL	HFG	3.1	1	1.2	403957
	13.5	CTS	TLGGIVYEQ	YFG	3.7	68	82.9	403957
	14	CAS	SERFGGDGNNPL	YFG	3.3	6	7.3	403957
	14	CAS	SPQFGGGHPTQIQ	YFG	3.5	1	1.2	403957

Sequences of TRB repertoires specific for the three defined Tp2 epitopes in A10-homozygous cattle are shown in the standardised format: identified Vβ and Jβ gene segments, flanking sequences of CDR3β and CDR3β itself. Number and frequency of encoding nucleotide sequences for each clonotype are shown. Dominant clonotypes for each of the nine CD8 T cell populations are shaded. Amino acid variants within germline gene component of CDR3β region are in red font and variants within junctional region of CDR3β region are in purple font and underlined. Germline Jβ gene components of CDR3β are underlined. Germline Dβ gene components of CDR3β are in blue font. Sequences with the same Vβ and Jβ gene segments but different CDR3β sequences are in brown font. The one clonotype identified from both $Tp2_{49-59}$ and $Tp2_{50-59}$ epitope-specific CD8 T cell populations from animal 403957 is in green font. Nucleotide sequences for the listed clonotypes are presented in Appendix B.

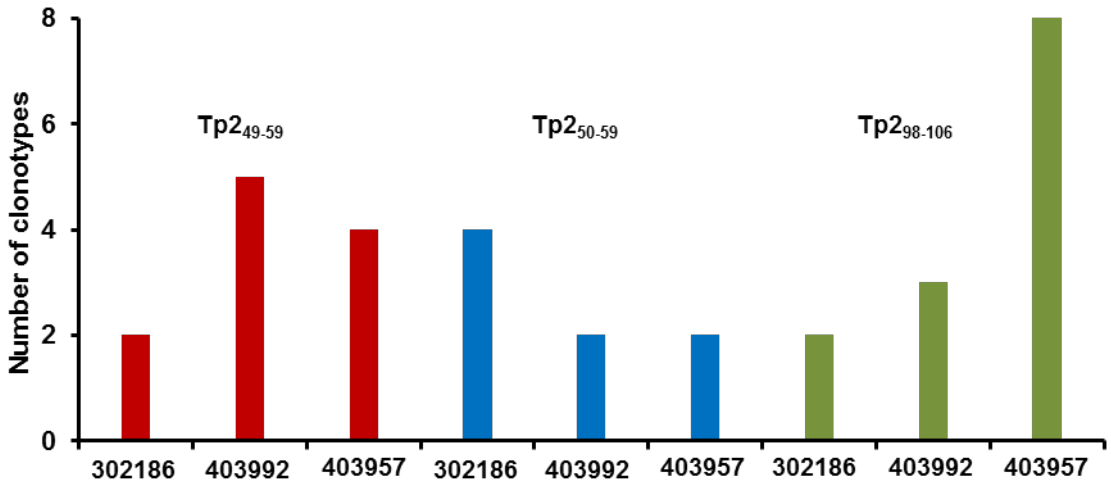


Figure 4.3 Number of TRB clonotypes confirmed for Tp2 epitope-specific CD8 T cell populations from A10 homozygous cattle.

Two to five TRB clonotypes were identified from 8 Tp2 epitope-specific CD8 T cell populations (3 populations in red bars for the $Tp2_{49-59}$ epitope, 3 populations in blue bars for the $Tp2_{50-59}$ and 2 populations in green bars for the $Tp2_{98-106}$ epitope). For one CD8 T cell population specific for the $Tp2_{98-106}$ epitope, from animal 403957 (green bar), 8 clonotypes were confirmed.

4.3.3 TRB repertoires of $Tp2_{98-106}$-specific CD8 T cells from A10 heterozygous cattle

Specific CD8 T cells were detected for only one of the 3 Tp2 epitopes ($Tp2_{98-106}$) in four out of six A10-heterozygous animals, one animal (402145) showing a very low percentage of epitope-specific CD8 T cells. $Tp2_{98-106}$ epitope-specific CD8 T cells were purified from the three A10-heterozygous cattle that showed readily detectable responses and analyses of their TRB repertoire were undertaken, using the same approach as described above, to examine their clonal diversity. As shown in Figure 4.4, diversity profiles for TRB clonotypes similar to those observed in A10-homozygous animals were observed, with a limited number of clonotypes detected in each animal and marked expansion of one dominant clonotype for each of the three purified CD8 T cell populations.

A striking feature of the results was that the dominant TRB clonotype in each of the 3 animals, which constituted 46-98% of sequences, used the same Vβ gene segment (Vβ13.5). Sequences with Vβ4.3 gene were also shared by clonotypes from two of the animals (102121 and 402082). Sequences for all identified TRB clonotypes are shown in Table 4-3. For clonotypes from different animals that had the same Vβ gene, the Jβ gene segments and CDR3β sequences were different. Overall, the numbers of identified clonotypes are 5, 2 and 4 for animals 102121, 102170 and 402082 respectively. Notably, the Vβ13.5 gene was also dominantly used by the $Tp2_{98\text{-}106}$ epitope-specific CD8 T cells from two of the A10-homozygous cattle (302186 and 403957).

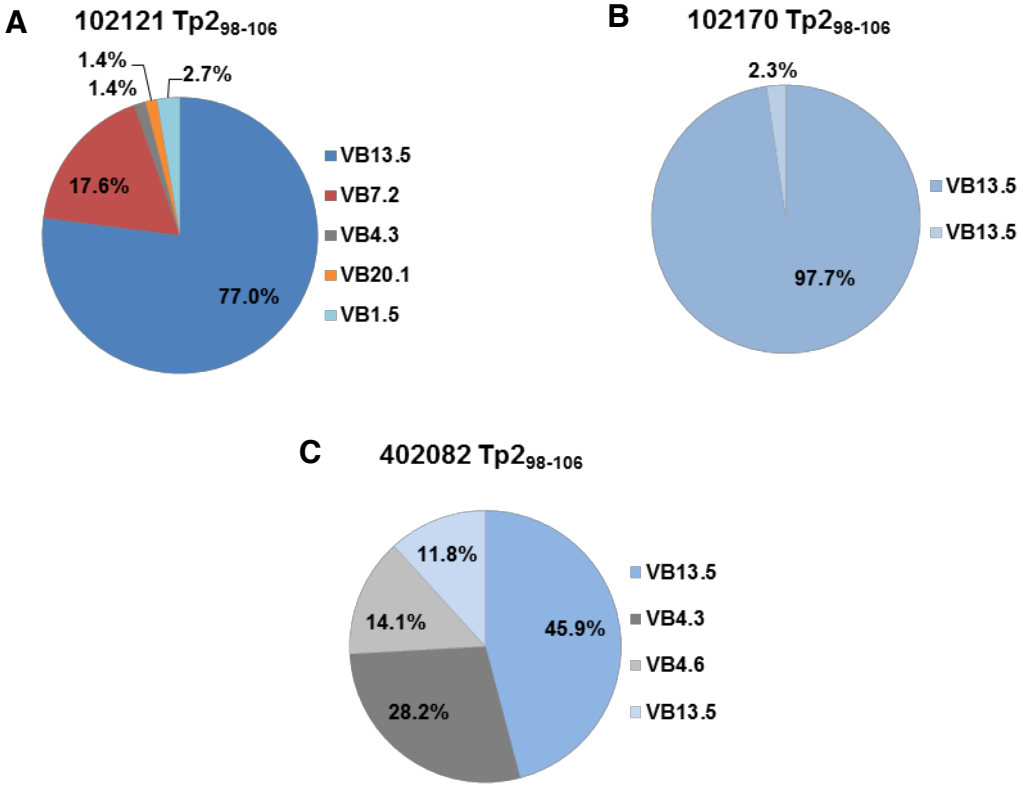


Figure 4.4 TRB clonotype distributions for Tp2 epitope-specific CD8 T cell populations obtained from A10 heterozygous cattle.

TRB clonotypes for each of the three $Tp2_{98\text{-}106}$ epitope-specific CD8 T cell populations obtained from A10-heterozygous cattle 102121, 102170 and 402082 are presented in A, B and C respectively. Each clonotype is represented by a unique colour. Vβ gene segments for identified clonotypes are shown. Clonotypes with the same Vβ gene usage but different CDR3β and/or Jβ genes are shown in different colours. Percentage of nucleotide sequence reads for each clonotype is illustrated.

Table 4-3 Sequences of TRB repertoires for $Tp2_{98-106}$ epitope-specific CD8 T cells from A10 heterozygous cattle

Epitope	Sequence					Number	Freq (%)	Animal
	Vβ	FR	CDR3	FR	Jβ			
$Tp2_{98-106}$	1.5	CAS	SEYPRGGHSNPL	YFG	3.3	2	2.7	102121
	4.3	CSA	GSWESETL	YFG	2.4	1	1.4	102121
	7	CAS	SSGFGGDDTQ	YFG	3.4	13	17.6	102121
	13.5	CTS	SLGGPYSETL	YFG	2.4	57	77.0	102121
	20.1	CAW	TSGGWNNPL	YFG	3.3	1	1.4	102121
	13.5	CTS	SLSGETL	YFG	2.4	2	2.3	102170
	13.5	CTS	NLGGITDTQ	YFG	3.4	84	97.7	102170
	4.3	CSA	PGTEGYEQ	YFG	3.7	24	28.2	402082
	4.6	CSA	GGDSYEQ	YFG	3.7	12	14.1	402082
	13.5	CTS	SLDSLRGY	HFG	1.2	10	11.8	402082
	13.5	CTS	SQDPHSGGNTQPL	YFG	3.2	39	45.9	402082

Sequences of TRB repertoires specific for the $Tp2_{98-106}$ epitope in A10 heterozygous cattle are shown in the standardised format: identified Vβ and Jβ gene segments, flanking sequences of CDR3β and CDR3β itself. Number and frequency of encoding nucleotide sequences for each clonotype are shown. Dominant clonotypes for each of the three CD8 T cell populations are shaded. Nucleotide sequences of listed clonotypes can be found in Appendix B.

In summary, analyses of samples of the TRB repertoires of Tp2 epitope-specific CD8 T cells in both A10-homozygous and -heterozygous cattle detected a similar profile of restricted number of TRB clonotypes specific for all three defined Tp2 epitopes - $Tp2_{49-59}$, $Tp2_{50-59}$ and $Tp2_{98-106}$. Each response was dominated by one or two highly abundant clonotypes. With one exception, which may relate to tetramer cross-reactivity, the TRB repertoires of T cells specific for each of the 3 Tp2 epitopes had distinct sequences and in most cases used different Vβ genes. However, the same Vβ gene segment was used by dominant clonotypes in $Tp2_{98-106}$-specific CD8 T cells in 5 of the 6 A10 animals that responded to this epitope.

Further analyses of the CDR3β sequences of T cells specific for each epitope were undertaken to investigate whether the TRB repertoires of the responding T cell in different animals exhibited any common features.

4.3.4 Detailed sequence analysis identified a conserved CDR3β motif in $Tp2_{98-106}$ epitope-specific CD8 T cells

Structural studies of TCR-pMHC I complexes have shown that the CDR3 regions of TCR make direct contact with peptide presented by MHC I molecules [Stewart-Jones et al., 2003, Garcia et al., 1999]. The data presented in Table 4-2 and Table 4-3 demonstrate that the CDR3β sequences of TRB clonotypes specific for the same Tp2 epitope vary in length and residue usages, without any obvious conservation of sequence. However, further analysis of the sequences of dominant clonotypes (sequences are summarized in Table 4-4) identified an "LGG" motif, which was present in the CDR3β sequences of dominant clonotypes identified in $Tp2_{98-106}$ epitope-specific responses of two A10-homozygous cattle (302186 and 403957) and two A10-heterozygous cattle (102121 and 102170), as shown in Figure 4.5. Additionally, these clonotypes utilize the same Vβ13.5 gene segment. The CDR3β sequences of CD8 T cells specific for $Tp2_{49-59}$ and $Tp2_{50-59}$ did not show such a feature.

Further analysis confirmed that the two glycines (GG) in the motif were encoded by a germline Dβ gene segment (sequence segment in blue font in Table 4-4), whereas the leucine (L) residue was encoded by a codon generated by non-germline nucleotide deletions/additions during TRB recombination.

Table 4-4 Sequences of dominant TRB clonotypes identified for the three Tp2 epitopes from both A10-homozygous and heterozygous cattle

Epitope	Sequence					Number	Freq (%)	Animal
	Vβ	FR	CDR3β	FR	Jβ			
$Tp2_{49-59}$	16	TGTGCCAGC C A S	AGCCTACAGCCTTTCTATGACTAT S L Q P F Y D Y	CACTTCGGC H F G	1.2	43	58.9	302186
	28	TGTGCCACG C A T	GCTGCAGATCTCGACGACAACCCTCTG A A D L D D N P L	TATTTTGGA Y F G	3.3	46	55.4	403992
	7.2	TGCGCCAGC C A S	AGTAGAGATCTAGTCGCAGAGACGCTG S R D L V A E T L	TACTTCGGC Y F G	2.4	23	44.2	403957
$Tp2_{50-59}$	28	TGTGCCAGC C A S	GCTGAATATGGGGGGGAGAACACCCAGCCCCTG A E Y G G E N T Q P L	TACTTTGGA Y F G	3.2	58	66.7	302186
	5	TGTGCCAGC C A S	GTCTCGCCTGGTGGGGACTAT V S P G G D Y	CACTTCGGC H F G	1.2	83	98.8	403992
	12.1	TGCGCCCAG C A Q	CATTCTTCGCGGGAGCAG H S S R E Q	TATTTCGGC Y F G	3.7	58	92.1	403957
$Tp2_{98-106}$	13.5	TGTACCAGC C T S	AATTTGGGGGGCCTGGACCTGGGCACTCAG N L G G L D L G T Q	TACTTCGGC Y F G	2.3	72	98.6	302186
	4.3	TGCAGTGCT C S A	GGTTCGGGCTATGAGCAG G S G Y E Q	TATTTCGGc Y F G	3.7	42	71.2	403992
	13.5	TGTACCAGC C T S	ACATTGGGGGGGATCGTCTATGAGCAG T L G G I V Y E Q	TATTTCGGC Y F G	3.7	68	82.9	403957
	13.5	TGTACCAGC C T S	AGTTTGGGGGGTCCGTACTCAGAGACGCTG S L G G P Y S E T L	TACTTCGGG Y F G	2.4	57	77.0	102121
	13.5	TGTACCAGC C T S	AACCTGGGGGGCATCACAGACACGCAG N L G G I T D T Q	TACTTCGGC Y F G	3.4	84	97.7	102170
	13.5	TGTACCAGC C T S	AGTCAGGACCCGCATTCGGGAGGGAACACCCAGCCCCTG S Q D P H S G G N T Q P L	TACTTTGGA Y F G	3.2	39	45.9	402082

Both nucleotide and amino acid sequences for dominant TRB clonotypes for the three Tp2 epitopes are shown in the standardised format: identified Vβ and Jβ gene segments, flanking sequences of CDR3β and CDR3β itself. Number and frequency of encoding nucleotide sequences for each clonotype are shown. Germline Jβ gene components in CDR3β are underlined and germline Dβ gene components are in blue font.

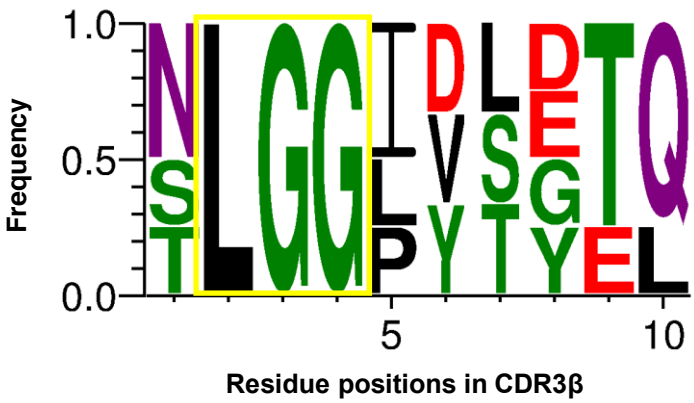


Figure 4.5 Preferential usage of a "LGG" motif by CDR3β sequences of dominant clonotypes specific for the Tp2$_{98-106}$ epitope from animals 302186, 403957, 102121 and 102170.
Colours represent physicochemical properties: polar (G, S, T, Y and C), green; neutral (Q and N), purple; basic (K, R and H), blue; acidic (D and E), red; hydrophobic (A, V, L, I, P, W, F and M), black. Residue size is proportional to frequency appeared in all aligned sequences. Graph was generated using a web-based application WebLogo3 (http://weblogo.threeplusone.com/).

4.3.5 Sharing of an identical TRB clonotype by the Tp2$_{49-59}$–specific response in different A10-homozygous animals

A previous study of TRB sequences of Tp2-specific CD8 T cells in two A10-homozygous cattle [Connelley et al., 2011] reported a conserved TRB rearrangement (Vβ28-CASAEYGGENTQPLYF-Jβ3.2) in the response to the Tp2$_{49-59}$ peptide in both animals. At that time, pMHC I tetramers were not available - in this study this TRB chain was identified within the Tp2$_{50-59}$ epitope-specific T cell populations of one of the A10-homozygous animals - 302186 (Table 4-2, the bold and italic sequence).

To verify the epitope specificity of CD8 T-cells expressing this TRB chain and to examine if it was shared in all Tp2$_{50-59}$-specific responses, a PCR to specifically amplify Vβ28-Jβ3.2 rearranged TRB chains was designed. Amplification of cDNA from Tp2$_{49-59}$ and Tp2$_{50-59}$ epitope-specific CD8 T cells isolated from 302186 using this PCR produced a band of the correct size from the Tp2$_{50-59}$-specific CD8 T cells

but not $Tp2_{49-59}$-specific CD8 T cells (Figure 4.6). Sequencing of the PCR product confirmed the Vβ28-CASAEYGGENTQPLYF-Jβ3.2 rearrangement.

Using the same method, the same TRB rearrangement Vβ28-CASAEYGGENTQPLYF-Jβ3.2 was also identified within $Tp2_{50-59}$ epitope-specific CD8 T cells from the other two homozygous cattle, 403992 and 403957 (Table 4-5). The clonotype from animal 403992 showed one nucleotide variant in the CDR3β region, which did not cause an amino acid coding change.

Together these findings demonstrated the existence of a shared or 'public' TRB chain present in the $Tp2_{50-59}$ epitope-specific CD8 T cell response of all 5 A10-homozygous animals examined.

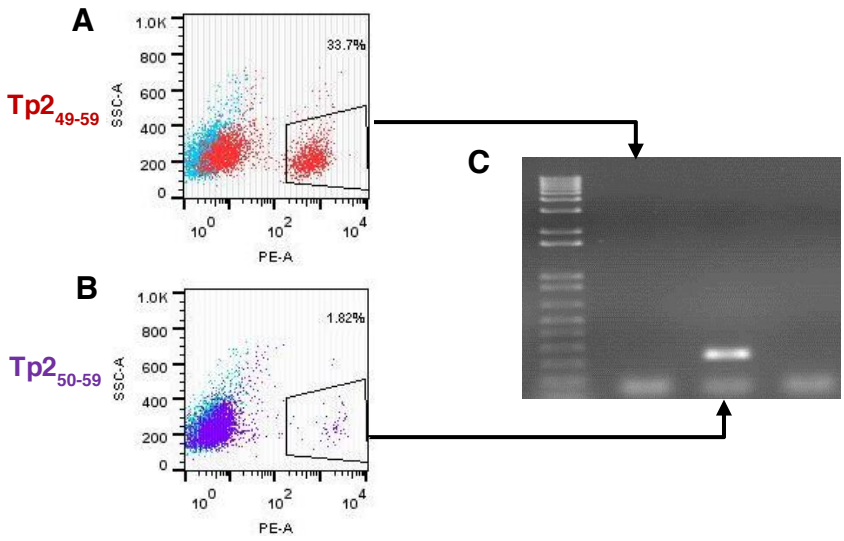


Figure 4.6 The Vβ28- CASAEYGGENTQPLYF-Jβ3.2 rearrangement was verified in $Tp2_{50-59}$ epitope-specific CD8 T cells from A10-homozygous cattle.

Both $Tp2_{49-59}$ (A) and $Tp2_{50-59}$ (B) epitope-specific CD8 T cells were sorted using FACS with >98% purity from CD8 T cell enriched cell line of A10-homozygous cattle (data shown from animal 302186). Unstained cell populations are shown in blue dots. $Tp2_{49-59}$ and $Tp2_{50-59}$ epitope-specific tetramer stained cells are shown in red and purple dots respectively. PCR amplification using Vβ28 and Jβ3.2 gene specific primers (see section 4.2.3) showed expected product from $Tp2_{50-59}$ epitope-specific CD8 T cells but not from $Tp2_{49-59}$ epitope-specific CD8 T cells (C).

Table 4-5 The TRB rearrangement Vβ28- CASAEYGGENTQPLYF-Jβ3.2 was identified from two A10-homozygous cattle 403992 and 403957

Epitope	sequence				Animal
	Vβ	FR	CDR3	FR	Jβ	
$Tp2_{50-59}$	28	TGTGCCAGC C A S	GCCGAATATGGGGGGGAGAACACCCAGCCCCTG A E Y G G E N T Q P L	TACTTTGGA Y F G	3.2	403992
	28	TGTGCCAGC C A S	GCTGAATATGGGGGGGAGAACACCCAGCCCCTG A E Y G G E N T Q P L	TACTTTGGA Y F G	3.2	403957

Both nucleotide and amino acid sequences for the identified TRB rearrangement Vβ28-CASAEYGGENTQPLYF-Jβ3.2 are shown in the standardised format: identified Vβ and Jβ gene segments, flanking sequences of CDR3β and CDR3β itself. Epitope specificity for the identified clonotype and the animals from which the clonotypes were identified are shown.

4.3.6 Epitope-specific TRB clonotypes in cultured CD8 T cells are representative of *in vivo* responding populations

The TRB repertoire data described above was derived from CD8 T cells that had been expanded *in vitro* by stimulation with irradiated parasitized cells. To investigate whether the clonotypic composition of these cell populations reflects the actual populations responding to parasite challenge *in vivo*, a preliminary experiment was undertaken to compare the TRB sequences of epitope-specific CD8 T cells obtained *ex vivo* from one of A10-homozygous immune animals (302186) with those obtained from *in vitro* cultures from the same animal. CD8 T cells were examined at the peak time point of the $Tp2_{49-59}$-specific CD8 T cell response (determined by tetramer staining - see section 3.3.6) after parasite challenge of the immune animal. Based on the previous observation that flow cytometric sorting of enriched tetramer sorted populations enabled the SMART-PCR to be used successfully, epitope-specific CD8 T cells were initially enriched by MACS cell purification of tetramer-stained cells. As shown in Figure 4.7, this increased the frequency of epitope-specific CD8 T cells (in red quadrats) from 1.46% to 65%. Flow cytometric sorting was then used to obtained a highly purified (≥98%) epitope-specific CD8 T cell population.

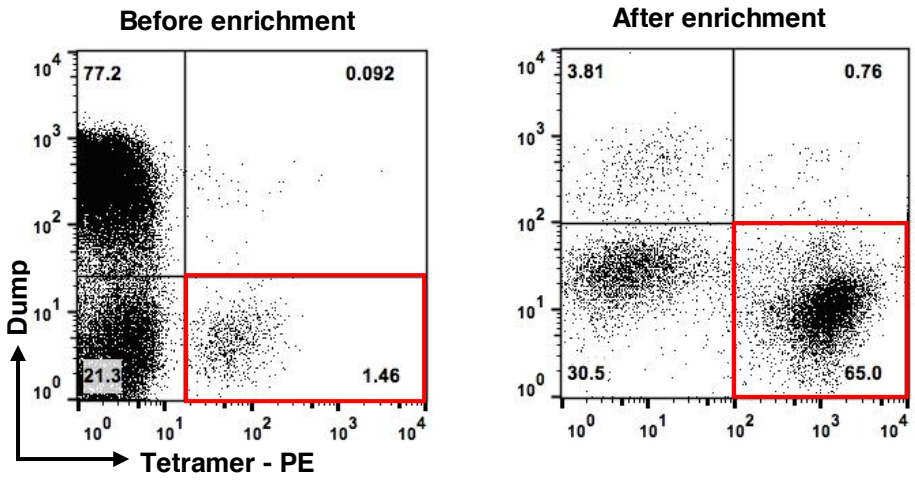


Figure 4.7 MACS enrichment of tetramer positive CD8 T cells. PBMC from *T. parva* challenged animal were stained with $Tp2_{49\text{-}59}$ tetramer and subjected to MACS sorting as described in section 4.2.1. Abundance of tetramer positive CD8 T cells increased from 1.46% in PBMC to 65% after enrichment. Percentage of epitope-specific CD8 T cells in the whole PBMC was determined through both tetramer and antibody staining as in section 3.3.6.

Sequences of TRB transcripts obtained from the *ex vivo* purified (≥98% purity) $Tp2_{49\text{-}59}$ epitope-specific CD8 T cells for animal 302186 are shown in Table 4-6. Three clonotypes observed *in vitro* were also observed from *ex vivo* purified epitope-specific CD8 T cells. The dominant clonotype (shaded) with 59% of sequencing reads within the *in vitro* data was also the most abundant clonotype (85% of sequencing reads) in *ex vivo* purified responding CD8 T cells. However, another highly abundant clonotype with the Vβ14-CASSRYAGPETLYF-Jβ2.4 rearrangement (34%, in italic font) in *in vitro* data was represented only at a low level (2.4%) within *ex vivo* population. Three clonotypes which were not present in *in vitro* data were identified from *ex vivo* purified epitope-specific CD8 T cells. These results indicated that the dominant TRB clonotypes identified in the *in vitro* cultured CD8 T cells are also present in the *in vivo* responding population, although their percentage representation in the two CD8 T cell populations differed. Further

depth of sequencing is required to more comprehensively compare the *in vitro* and *in vivo* repertoires (see further discussion below).

Table 4-6 TRB repertoires specific for the Tp2$_{49-59}$ epitope from both *in vitro* memory and *ex vivo* responding CD8 T cells

Epitope	Sequence					*In vitro*		*Ex vivo*	
	Vβ	FR	CDR3	FR	Jβ	Number	Freq(%)	Number	Freq(%)
	4.6	CSA	EGLGSL	YFG	3.3	0	0	1	2.4
	13.2	CAS	SYGTGYEQ	YFG	3.7	0	0	1	2.4
	14	CAS	SEGNSNYDY	HFG	1.2	0	0	2	4.9
Tp2$_{49-59}$	*14*	*CAS*	*SRYAGPETL*	*YFG*	*2.4*	*25*	*34.2*	*1*	*2.4*
	16	CAS	SLQPFYDY	HFG	1.2	43	58.9	35	85.4
	16	CAS	SLQPFYVY	HFG	1.2	4	5.5	0	0
	16	CAS	SLQPFYGY	HFG	1.2	1	1.4	1	2.4

Sequences of TRB repertoires specific for the Tp2$_{49-59}$ epitope obtained from both *in vitro* cultured (derived from immune cattle 302186) and *ex vivo* purified (from parasite challenged cattle 302186) CD8 T cells are shown in the standardised format: identified Vβ and Jβ gene segments, flanking sequences of CDR3β and CDR3β itself. Number and frequency of encoding nucleotide sequences for each clonotype are shown. The dominant clonotype is shaded. The clonotype in italic font shows significant decrease of abundance in the *ex vivo* data. Nucleotide sequences of listed clonotypes can be found in Appendix B.

4.4 Discussion

Work presented in the current chapter described the assessment of TRB repertoires for the three defined Tp2 epitopes and the potential relevance of repertoire usage to the epitope dominance hierarchy and the capacity of CD8 T cell responses to recognize allelic variants of epitopes. Meanwhile, the identified TRB clonotypes were analysed to determine whether there are similarities between animals in the TRB clonotypes of CD8 T cells recognizing the same epitopes.

During TCR recombination, germline gene segments rearrangement and non-germline nucleotide additions/deletions at gene junctions result in a diverse repertoire of αβTCR [Nikolich-Zugich et al., 2004]. The sequence of the junctional region provides a unique marker for each rearrangement and this region also encodes the CDR3 regions of the TCR polypeptide chains. In the case of TCRβ chain, the protein loop encoded by the CDR3 region interacts closely with the MHC-bound peptide and hence is important in determining antigenic specificity [Turner et al., 2006]. TRB sequencing has been used as an approach of analysing clonotypic composition of CD8 T cell responses [Koning et al., 2013, La Gruta et al., 2008, Kedzierska et al., 2006]. For a defined epitope, CD8 T cells can utilise a range of different TCRβ chains incorporating different gene segments and with different CDR3 sequences. The TRB clonotypes of a particular epitope usually vary between different animals, which are referred to as private repertoires [Kim et al., 2005]. However, TRB rearrangements that are conserved between animals have also been observed for CD8 T cell responses to some epitopes and are named as public repertoires [Venturi et al., 2008a, Venturi et al., 2006, Kedzierska et al., 2004]. These public TRBs can serve as useful markers to monitor the respective epitope specific CD8 T responses.

Previous studies analysed TRB sequences of CD8 T cells from two A10-homozygous cattle reactive with the $Tp2_{49-59}$ epitope [Connelley et al., 2011]. However, the $Tp2_{50-59}$ epitope was not defined at that time and the TRB sequences were obtained from a panel of epitope-specific CD8 T cell clones derived from CD8 T cell cultures subjected to 3 *in vitro* stimulations. The approach used for TRB

analysis, involving CD8 T cell cloning and Vβ subfamily-specific PCR and sequencing, is time-consuming and limits the number of epitope-specific CD8 T cell populations that can be analysed [Connelley et al., 2008]. A template-switch anchored RT-PCR, coupled with pMHC I tetramer staining and MACS/FACS purification techniques, allows direct TRB repertoire analysis of uncloned epitope-specific CD8 T cells [Quigley et al., 2011]. Moreover, attempts were made to obtain epitope-specific CD8 T cells from primary CD8 T cell cultures, in order tominimise the potential alteration of clonotype compositions due to prolonged *in vitro* culture [Koning et al., 2014, Belyakov et al., 2001]. Additionally, attempts were also made to isolate epitope-specific CD8 T cells directly *ex vivo* from one parasite challenged immune animal.

A common feature of TRB clonotypes for the examined epitope-specific CD8 T cell populations, derived from both *in vitro* cultured CD8 T cells and PBMCs isolated *ex vivo*, was the presence of one or two expanded clonotypes that accounted for a large proportion of the sequences obtained. The detection of only two clonotypes in 63-86 sequencing reads obtained for 5 of the Tp2 epitope-specific CD8 T cell populations ($Tp2_{49-59}$-specific CD8 T cells from 302186, $Tp2_{50-59}$-specific CD8 T cells from 403992 and 403957, $Tp2_{98-106}$-specific CD8 T cells from 302186 and 102170) are extreme examples of this. Many CD8 T-cell responses are characterised by a clonotypic structure in which there are a limited number of dominant clonotypes and a large number of very low frequency clonotypes [Chen et al., 2001, Naumov et al., 1998]. A comprehensive analysis of the full TRB repertoire would therefore require a substantially greater depth of sequencing than was attempted in this study, where the primary aim was to characterise the more abundant clonotypes.

As with any TRB analysis conducted on *in vitro* CD8 T-cells, it is possible that certain clonotypes are preferentially expanded during the period in culture, leading to a clonotypic profile that is not representative of the *in vivo* population. To address this, an experiment comparing the TRB repertoires for both *in vitro* cultured CD8 T cells and *ex vivo* CD8 T cells isolated during the active response to parasite challenge from the same animal (302186) was performed. Of the 4 clonotypes identified *in vitro* and 6 clonotypes identified in the *ex vivo* population, 3 were common to both.

In both populations the same TRB clonotype (Vβ16-CASSLQPFYDYHF-Jβ1.2) was dominant (85% *in vivo* and 59% *in vitro*) and the second dominant clonotype (Vβ14-CASSRYAGPETLYF-Jβ2.4) in the *in vitro* memory population was also evident in the *in vivo* responding CD8 T cells, although at a lower frequency (2.4% *in vivo* and 34% *in vitro*). Notably, the clonotypes that appeared to be unique to either the *in vitro* or *ex vivo* populations were present at low frequency, suggesting the failure to find them in the other population may have been an issue of resolution that could be resolved by sequencing more TRB chains. These results are consistent with previous findings [MacHugh et al., 2009], where remarkably similar clonotypic profiles were demonstrated for *in vitro* and *ex vivo T. parva*-specific CD8 T cell responses using the TRB heteroduplex technique.

The large component of these dominant TRB clonotypes in epitope-specific CD8 T cells indicates their major contribution to the fine antigenic specificity of CD8 T cell responses against *T. parva*. In a murine influenza virus model, TCRβ chain clonotypes were demonstrated to determine the avidity of CD8 T cells to viral epitopes [Moffat et al., 2010]. Selective expansion of high-avidity CD8 T cells, which has been reported in both human and mouse anti-virus responses [Cukalac et al., 2014a, Price et al., 2005], could be responsible for the clonal expansion of few dominant clonotypes observed in the current study.

Previous studies of CD8 T cell responses against the $Tp2_{49\text{-}59}$ epitope, using synthetic peptides with amino acid substitutions, have shown that different TRB clonotypes can vary in their ability to recognise peptides with substitutions at some positions [Connelley et al., 2011]. Hence, CD8 T cell responses with a very narrow TRB repertoire may be less likely to cross-react with epitope variants containing one or two amino acid substitutions. The polymorphism of Tp2 antigen of *T. parva* has been demonstrated, showing a total of 23 allelic variants for the $Tp2_{49\text{-}59}$ epitope from 82 examined parasite isolates [Pelle et al., 2011]. Future investigation to define the cross-reactivity of identified TRB clonotypes to allelic variants of Tp2 epitopes would be of help to examine the capacity of CD8 T cell responses against heterologous infection.

Sequence analysis of epitope-specific TRB clonotypes identified from different animals observed i) the preferential usage of Vβ13.5 gene and a semi-conserved CDR3β motif by the $Tp2_{98-106}$ epitope-specific CD8 T cells in 2 A10-homozygous and 3 A10-heterozygous cattle, ii) a conserved TRB clonotype with Vβ28-CASAEYGGENTQPLYF-Jβ3.2 rearrangement from $Tp2_{50-59}$ epitope-specific CD8 T cells in all examined A10-homozygous cattle. Reasons for the preferential usage of Vβ13.5 gene by the $Tp2_{98-106}$ epitope are unclear. However, extensive studies in human and mouse models suggested that both MHC I-restricted thymic selection and structural complementarity of TCR to its cognate peptide-MHC I complex can induce the biased usage of particular TCR V genes [Turner et al., 2006]. The conserved TRB clonotype usage by different animals has been reported in many virus disease models and proposed as a result of recombinatorial biases and convergent recombination during TCR rearrangement [Li et al., 2012, Venturi et al., 2008b]. Functionality of the conserved Vβ28-CASAEYGGENTQPLYF-Jβ3.2 clonotype is yet to be defined. However, the dominant status of this clonotype within the $Tp2_{50-59}$ epitope-specific CD8 T cells in animal 302186 indicated its rapid responding capacity against parasite infection. This is consistent with the previous observations in two A10-homozygous cattle, where a significant increase of proportions of the conserved clonotype within Tp2 antigen-specific CD8 T cells was observed after parasite challenge [Connelley et al., 2011].

Overall, profiles of TRB clonotypes for the three defined Tp2 epitopes were analysed in both A10-homozygous and -heterozygous cattle, providing a general view of clonotypic dominance in epitope-specific CD8 T cell populations. A conserved TRB rearrangement specific for the $Tp2_{50-59}$ epitope was verified, which serves as a marker of antigen-specific CD8 T cell responses, and most importantly, allows tracking of antigen-specific CD8 T cell responses *in vivo*. With the defined Vβ and Jβ gene segments, a more comprehensive repertoire diversity would be achieved employing high through-put sequencing approaches.

Chapter 5: Identification of $Tp2_{50-59}$-specific T-cells through high throughput sequencing analysis to detect a 'public' TCR

5.1 Introduction

In previous chapters, the observation of Tp2 epitope dominance variation between BoLA-A10-homozygous and heterozygous cattle suggests that bovine immunodominant CD8 T cell responses against *T. parva* can't be predicted solely based on the presence of MHC I restriction elements. T cell receptor β chain (TRB) repertoires for the three defined Tp2 epitopes were analysed for both BoLA-A10-homozygous and heterozygous cattle in the last chapter to investigate their potential contribution to the formation of the immunodominance hierarchy. From the obtained epitope-specific TRB clonotypes, a conserved $Tp2_{50-59}$ epitope-specific TRB rearrangement was verified to be shared by multiple BoLA-A10-homozygous individuals with unrelated genetic background. This conserved TRB sequence provides a unique 'barcode' for this epitope-specific CD8 T cell response, which can be exploited to further study the effect of the TRB repertoire on the immunodominance of epitopes of cattle CD8 T cell responses against *T. parva*.

Altered hierarchy of antigen-specific CD8 T cell responses in MHC class I diversified individuals have been reported previously in mice and human virus disease models [Betts et al., 2000, Belz et al., 2000]. In an influenza virus mouse model, the CD8 T cell response against some epitopes was reduced by up to 95% in F1 progeny compared to their inbred parents [Day et al., 2011]. In the case of human immunodominant CD8 T cell responses against human immunodeficiency virus (HIV), more complex situations have been observed. Epitope dominance varies greatly between individuals having defined MHC class I restriction elements, with CD8 T cell responses against some epitopes only present in a subset of individuals [Betts et al., 2000]. Potential factors that could influence both antigen processing and CD8 T cell activation were examined to understand the mechanistic basis of this phenomenon [Day et al., 2011, Flesch et al., 2010]. However, a definitive conclusion wasn't achieved due to the complexity of the parameters that influence

immunodominance [Yewdell, 2006]. In spite of this, antigen-specific TCR repertoire changes during ontogeny were proposed as a potential reason, if not the complete explanation, for the diminished epitope specificity in MHC I heterozygotes. Similarly, in the murine influenza virus model, loss of a prominent epitope-specific TRB repertoire in $H2^{k\times b}$ F1 mice was reported to be responsible for the decreased epitope-specific CD8 T cell responses when compared with their inbred parents [Belz et al., 2000]. In a human Epstein-Barr virus (EBV) model, deletion of a dominant TCR specific for an HLA-B8 restricted EBV-derived epitope FLRGRAYGL was observed in heterozygous individuals with a cross-reacting alloantigen HLA-B*4402 [Burrows et al., 1995, Argaet et al., 1994]. Although individuals with HLA-B*4402 expression used diversified TCRs to recognize the epitope, this observation clearly demonstrates that presence of additional MHCI alleles can alter the epitope-specific TCR repertoire.

As presented in Chapter 3, Tp2 epitope dominance variation was observed for cattle with the MHC class I restriction allele 2*01201 (designated to the BoLA-A10 haplotype). For BoLA-A10-homozygous cattle, CD8 T cells showed dominant responses to the $Tp2_{49\text{-}59}$ epitope, with subdominant responses to the $Tp2_{50\text{-}59}$ and $Tp2_{98\text{-}106}$ epitopes. For BoLA-A10-heterozygous cattle, the $Tp2_{98\text{-}106}$ epitope became dominant in some but not all animals; and CD8 T cell responses to the other two epitopes were not detectable in any of the heterozygous cattle. Reasons for Tp2 epitope dominance change in BoLA-A10-heterozygous cattle are unclear. The results presented in Chapter 3 clearly indicate that the presence of other MHC class I alleles has negative effects on CD8 T cell responses against $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitopes. Evidence for the presentation of the $Tp2_{49\text{-}59}$ epitope by parasite-infected BoLA-A10-heterozygous cells is available from previous studies, where *T. parva* specific CD8 T cells generated through autologous stimulation showed specific CD8 T cell response to the $Tp2_{49\text{-}59}$ epitope for one BoLA-A10/A18 heterozygous animal [Graham et al., 2008]. Therefore, it was decided to examine if the absence of detectable $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$-specific responses in the six A10-heterozygous cattle used in this study was due to TCR repertoire alteration, leading to the deletion of T cells specific for these epitopes. Due to the diversity of the TCR repertoire and the generally 'private' nature

of epitope-specific T cells responses (where individuals in an outbred population will express unique TCRs in responses against defined epitopes) [Kedzierska et al., 2008], application of TCR analysis to address such questions can be difficult. However, the presence of the conserved TRBV28^{+} TRB rearrangement in the Tp2$_{50\text{-}59}$ epitope-specific responses provides a representative TRB sequence that could be used to investigate if Tp2$_{50\text{-}59}$ epitope-specific CD8 T cells are deleted in BoLA-A10 heterozygous animals.

Using an indirect approach, the diversity of an individual human's TRB repertoire was estimated to be ~10^{6} for naïve T cells and 1-2×10^{5} for memory T cells [Arstila et al., 1999]. Later approaches using high-throughput sequencing technology observed the same TRB diversity for naïve T cells but surprisingly a 10 to 20-fold larger TRB diversity for memory T cells than previous reports [Robins et al., 2009]. Although the bovine TRB repertoire diversity is yet to be determined, the available data indicates the presences in the genome of aminimum of 86 functional TRBV, 3 TRBD and 17 TRBJ genes. The potential VDJ permutations that can be used for functional TRB recombination were also estimated to be about 3 times of that for human [Connelley et al., 2009]. Detection of a defined TRB rearrangement in highly complex populations (e.g. quiescent PBMC) requires analysis that has the capacity to provide a high resolution TRB repertoire profile. Due to their inherent limitations of low throughput and low resolution, traditional methods of examining TRB repertoires such as Sanger sequencing and spectratyping are not suited to perform such analysis [Calis and Rosenberg, 2014, Six et al., 2013]. However, high-throughput sequencing (HTS) technologies are rapidly becoming powerful tools for TCR repertoire profiling [Six et al., 2013, Baum et al., 2012], and it was decided to attempt detection of the public TRBV28^{+} Tp2$_{50\text{-}59}$-specific CD8 T cell TRB chain directly *ex vivo* from CD8 T cell isolated from PBMC using this approach.

To validate this approach, an initial experiment was conducted to detect the public TRBV28^{+} Tp2$_{50\text{-}59}$-specific TRB chain from memory CD8 T cells of *T. parva*-immunised BoLA-A10-homozygous animal in which the public TRB chain had been detected during *in vitro* analyses. Once established the approach would then be used for subsequent detection of potential epitope-specific CD8 T cell clonotypes from the

naïve repertoire of BoLA-A10-homozyogus and heterozygous cattle, in order to determine if TRB repertoire alteration could contribute to the observed variation in the epitope dominance of CD8 T cell responses in the *T. parva* system.

5.2 Materials and methods

5.2.1 Sample preparation for Illumina sequencing

The detailed procedure for sample preparation is described in section 2.3.3. Briefly, CD8 T cells were purified from isolated PBMC from parasite immunized A10-homozygous cattle, according to the method described in section 2.2.2. Purified CD8 T cells were then checked for purity and CD4 T cell contamination using flow cytometry. Further purification, by flow cytometric sorting to eliminate CD4 T cells was performed if necessary. cDNA of purified CD8 T cells was prepared using the Promega® Reverse Transcription System as described in section 2.3.3.3. Forward and reverse primers to permit specific amplification of TRBV28^{+}-TRBJ2.2/3.2^{+} TRB chains (Table 5-1) were designed. Oligonucleotide adapters and indexes were added to the 5' end of gene-specific primers for sequencing and sample identification. Two reverse primers (only differing in the sample index sequence) were used to permit multiple-sample indexing. The obtained PCR amplicons were purified using AMpure Beads (Beckman Coulter, High Wycombe, UK) according to section 2.3.3.5 and the purity was checked using Agilent 2200 TapStation (Agilent Technologies) according to the manufacturer's instructions. The purified amplicons were then sent for sequencing on the Illumina® HiSeq2500 platform. Paired-end reads of 250bp in length for each direction were generated.

Table 5-1 Primers for CDR3 region amplification and Illumina sequencing.

Primer	Sequence
VB28F1	5'-AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTA CACGACGCTCTTCCGATCTgcgcaggatcttggagactga-3'
JBR1	5'-CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTcagcacagtcagcttggaac-3'
JBR2	5'-CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCTcagcacagtcagcttggaac-3'

Gene-specific forward (VB28F1) and reverse (JBR1 and JBR2) primers are shown. The regions in lowercase are specific for Vβ28 and Jβ2.2/3.2 segments. Regions in uppercase are adapters added to amplicons for sequencing and those underlined are identifier tags for sample identification.

5.2.2 Sequence data analysis

Sequence data obtained from the Illumina platform were analyzed according to published algorithms [Bolotin et al., 2012, Warren et al., 2011], modified to fit with the objective of this study. A flow chart in Figure 5.1 illustrates the major steps for data analysis. Two approaches were used for estimating the diversity of Vβ28-Jβ3.2 TRB rearrangements.

(i). Extract sequences with valid Vβ and Jβ gene segments. Since Vβ and Jβ gene segments have been defined during amplicon preparation, raw sequence reads with valid Vβ and Jβ nucleotide sequences were selected for further analysis. During this process, sequences with low-quality nucleotides (<Q30 quality score – probability of a 1 in 1000 incorrect base call) were removed.

(ii). Identify CDR3β. Sequences with valid Vβ and Jβ gene segments were then aligned for localizing Vβ and Jβ genes on each reads. CDR3β regions were defined through identification of the three nucleotides encoding the conserved amino acid Cys (C) located towards the 3' end of the V gene segment and the three nucleotides encoding the conserved amino acid Phe (F) in 5' end of the J gene segment.

(iii). Extract CDR3β sequences containing the Jβ3.2 gene segment. The specific reverse primer designed to amplify this gene rearrangement is within a 30bp region of Jβ3.2, from the 3' end to the conserved Phe-Gly-X-Gly (F-G-X-G) motif, which is conserved between Jβ2.2 and Jβ3.2 germline sequences. Transcripts containing Jβ2.2 were identified based on differences in sequences 5' to this conserved motif. This enabled the remaining CDR3β sequences containing Jβ3.2 and Vβ28 gene segments to be identified and grouped into unique CDR3β sequences.

(iv). Error rate calculation. In order to estimate the proportion of CDR3β that were potentially generated from artefacts (i.e. PCR or sequencing errors), sequencing reads were aligned with the defined Vβ gene segment to identify erroneous nucleotides. Error rate was calculated according to the following formula. Given the average length of CDR3β sequences, a cutoff for erroneous sequences was calculated as (error rate×CDR3β length (kbp) ×100) %. According to the cutoff, a respective

proportion of low-abundant sequences were removed tominimize all potential erroneous CDR3β sequences.

$$\text{Error rate (errors/kbp)} = \frac{\text{Total number of erroneous nucleotides in V}\beta}{\text{Total number of aligned sequences} \times \text{length of V}\beta\text{ (kbp)}}$$

(v). Clonotype identification. First, sequences representing "core clonotypes" were identified by grouping identical CDR3β nucleotide sequences. Second, correction of PCR and sequencing errors was performed through merging low-abundant "core clonotypes" with more abundant "core clonotypes" that differs by no more than three nucleotide mismatches within the germline V, D and J segments of CDR3. For the three nucleotide mismatches, ≤2 mismatches are allowed within the V gene segment (excluding the last two identified nucleotides), ≤2 mismatches are allowed within the J gene segment (excluding the first two identified nucleotides), ≤1 mismatches are allowed within the D gene segment (excluding the first two and the last two identified nucleotides). By doing this, diversity of junction sequences of VDJ will not be influenced. This correction is feasible as TCRs do not undergo somatic hypermutation and therefore mismatches within germline V, D and J segments of CDR3 can only arise from PCR or sequencing errors. Dβ genes are usually difficult to define due to nucleotide additions and deletions during somatic rearrangement. In the current study, error correction was conducted for two sample clonotypes with identifiable germline Dβ gene segment within CDR3β region. PCR or sequencing error rate was estimated according to these samples.

(vi). To determine the presence and frequencies of clonotypes with the defined CDR3β sequence, the filtered data were searched for the conserved epitope-specific TRB rearrangement Vβ28-CASAEYGGENTQPLYF-Jβ3.2.

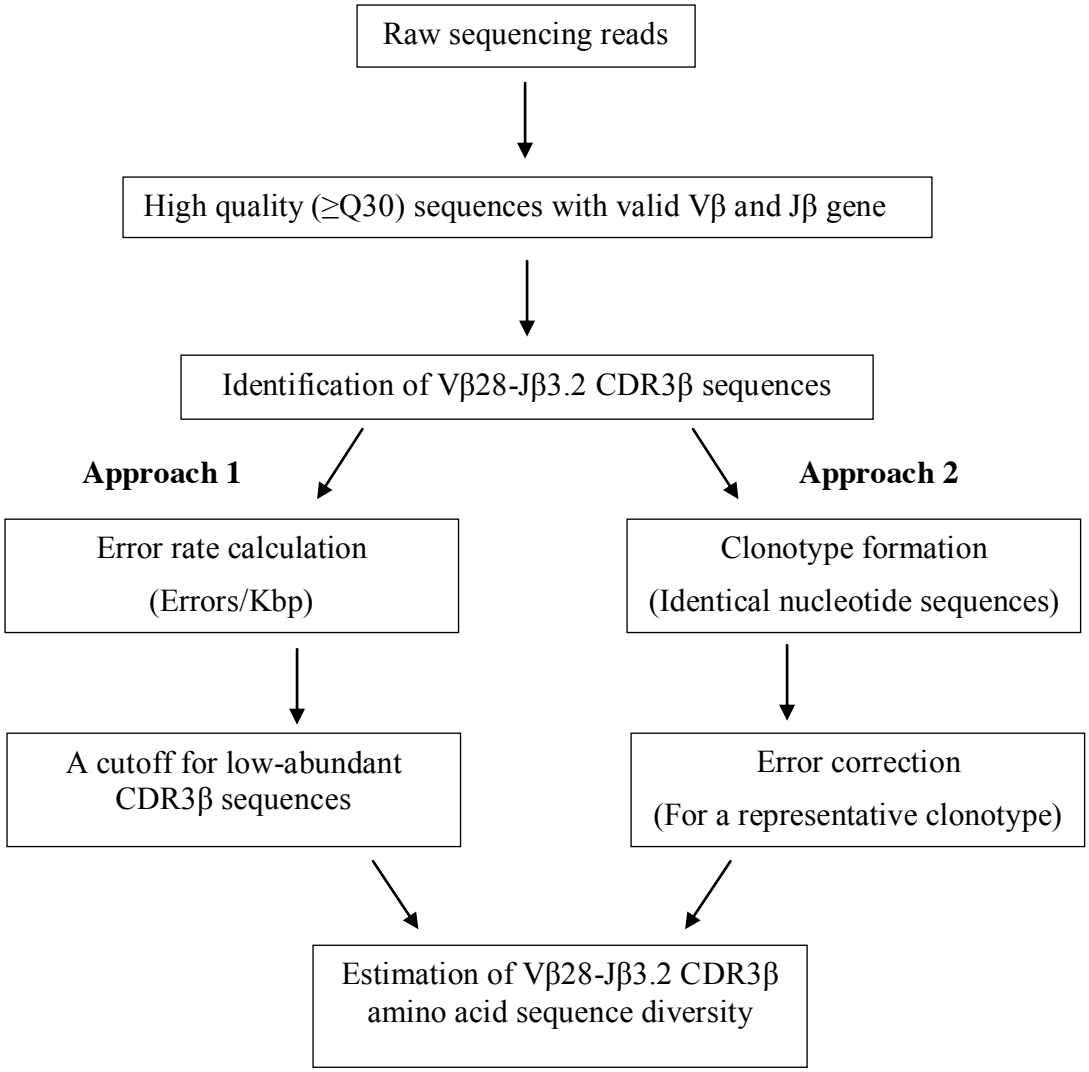


Figure 5.1 Flow chart for steps of sequencing data analysis. Raw sequencing reads were initially trimmed according to quality score (Q30) and Vβ and Jβ gene alignment. From the resultant high-quality sequencing reads, sequences with Vβ28-Jβ3.2 rearrangement were used for subsequent error correction according to two approaches described in section 5.2.2. An estimation of Vβ28-Jβ3.2 repertoire diversity was made based on the error analysis, which can indicate the depth of sequencing data.

5.3 Results

5.3.1 Amplicon preparation for Illumina sequencing

As described in the previous chapter, a conserved (or public) TRB sequence with a Vβ28-CASAEYGGENTQPLYF-Jβ3.2 rearrangement is shared by the $Tp2_{50-59}$ epitope-specific CD8 T cells from 5 BoLA-A10-homozygous cattle. Using this as a 'barcode' for $Tp2_{50-59}$ -specific CD8 T cells may allow HTS analysis of the TRB repertoire to be used for the detection of epitope-specific CD8 T cell clonotypes within highly diverse repertoires, such as observed in samples derived directly *ex vivo*. To maximize the chances of achieving sufficient sequencing depth to detect this TRB chain, PCR amplification using primers specific for the defined Vβ28 and Jβ3.2 genes were designed. As i) Vβ28 is a single member subgroup, and the bovine genome contains aminimum of ~90 functional TRBV genes and ii) the primers were specific for only 2 (Jβ 2.2 and 3.2) of the 17 functional bovine TRBJ genes [Connelley et al., 2009], it was estimated that compared to the pan-TRB amplification protocol described in previous chapters, similar sequencing levels using this PCR would enable an ~700 fold higher resolution of the repertoire of Vβ28-Jβ2.2/3.2 TRB rearrangement.

CD8 T cells purified from PBMC of a previously *T. parva* immunized BoLA-A10-homozygous animal (403957) were initially used to validate the methodology. Purity and CD4 T cell contamination of isolated CD8 T cells were checked. As shown in Figure 5.2A, purity of the CD8 T cells was above 97%. No significant CD4 T cell contamination was observed (data not shown). From two aliquots of purified CD8 T cells (1×10^7 cells per aliquot), TRB amplicons with the defined Vβ28 and Jβ2.2/3.2 gene segments were prepared using the gene-specific primers. High-fidelity DNA polymerase was used to limit PCR errors during amplification. PCR cycles were checked to obtain adequate amplicons for sequencing. As shown in Figure 5.2B, 30 PCR cycles were sufficient to generate a visible PCR product (length around 450bp). Purity of the product post-purification with AMpure beads was confirmed by agarose gel electrophoresis (Figure 5.2C). Paired-end reads with a length of 250bp for each direction were generated on an Illumina HiSeq2500 platform and forward and reverse reads were paired to get contiguous sequences, as shown in Figure 5.2D.

The quality parameters of generated sequences for both samples are shown in Figure 5.3. Quality scores for nucleotides upto the 250bp position was ≥Q30, a benchmark quality score for Illumina sequencing. Lower qualities were observed for nucleotides between 250-500bp. For data analysis, sequences with low-quality nucleotides were removed and only the remaining high-quality sequences were used.

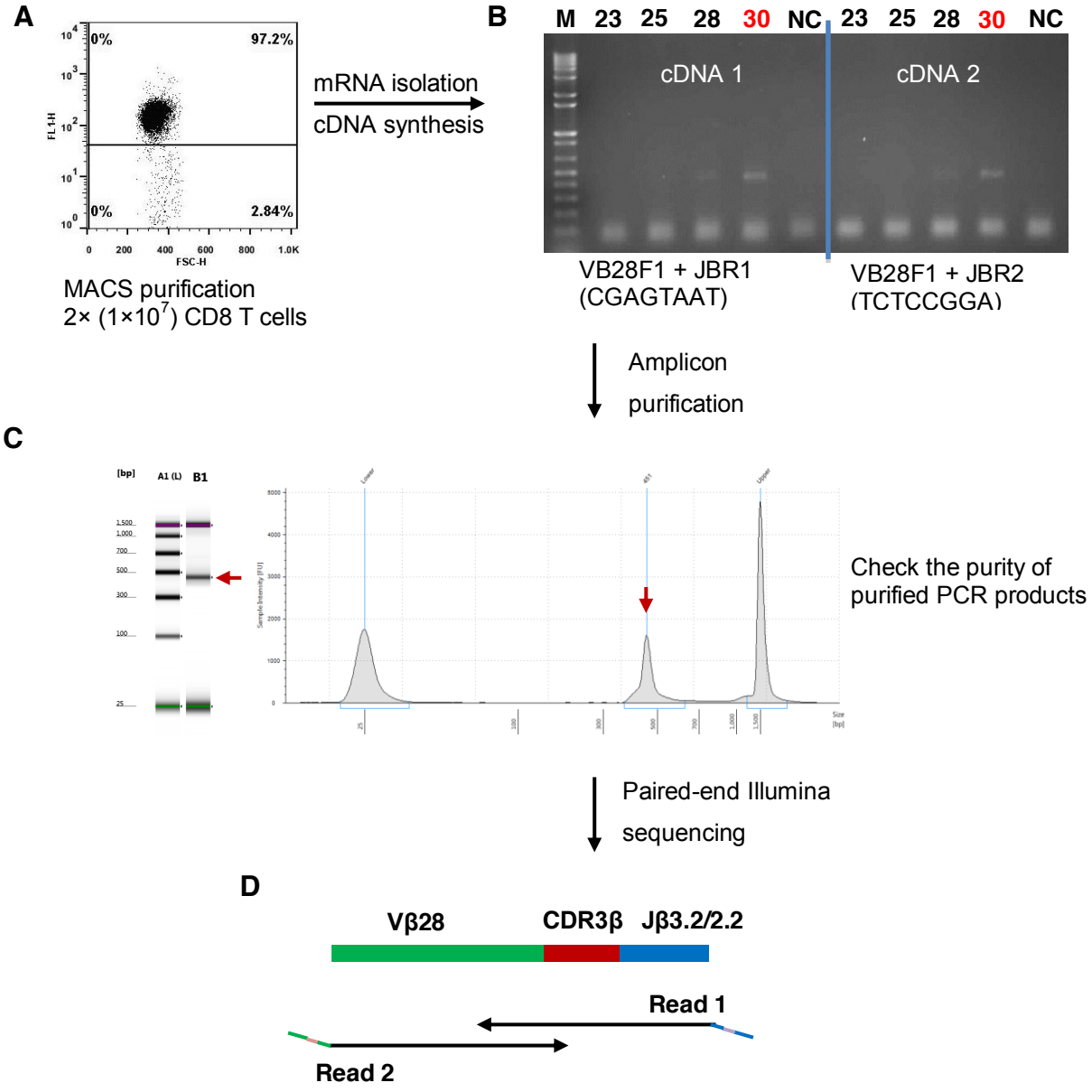


Figure 5.2 Preparation of Vβ28-Jβ2.2/3.2 TCRβ amplicons for Illumina sequencing.
A, CD8 T cells were purified from PBMC of *T. parva*-immunized BoLA-A10 homozygous animal 403957. Two aliquots of 1×10^7 CD8 T cells were used for amplicon preparation. B, PCR cycles were checked to choose the optimal condition for obtaining adequate amplicons for sequencing. C, PCR amplicons were purified using AMpure beads according to methods described in section 2.3.3.5 and the purity of the resultant PCR products were checked using Agilent 2200 TapStation. D, PCR amplicons were sent for sequencing on the Illumina® platform. Paired-end reads were generated for CDR3β clonotype analysis.

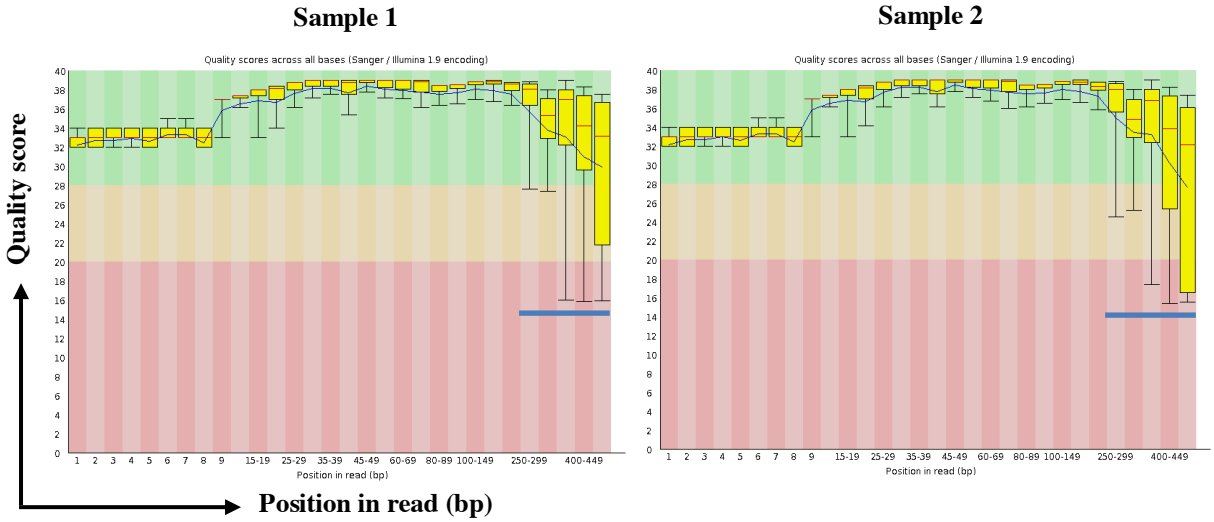


Figure 5.3 Quality of nucleotide sequences generated from Illumina sequencing. Quality scores across all nucleotide bases are shown for the raw data of both samples. For the two samples, nucleotides upto 250 bp position were ≥Q30. Quality scores for nucleotide bases marked by blue lines showed variation. Sequences with low quality scores were removed and the remaining high-quality sequences were used for subsequent analysis.

5.3.2 Clonal diversity of defined Vβ-Jβ gene rearrangements

5.3.2.1 Extraction of sequences with in-frame CDR3β

As shown in Table 5-2, for both samples, 91% of raw sequences had the defined Vβ and Jβ gene segments, providing 2.8 million sequences for sample 1 and 3.5 million for sample 2. CDR3β sequences were extracted by identification of the three nucleotides encoding the conserved amino acid Cys (C) located towards the 3' end of the V gene segment and the three nucleotides encoding the conserved amino acid Phe (F) in 5' end of the J gene segment. For samples 1 and 2, CDR3β sequences were obtained for ~2.6 million and ~3 million reads respectively, of which ~2 and ~2.4 million sequences were identified as being with Vβ28-Jβ3.2 rearrangements. Approximately 1×10^5 and 1.32×10^5 unique CDR3β amino acid sequences were encoded by the nucleotide sequences in samples 1 and 2 respectively. CDR3β length distributions for the identified unique amino acid sequences are shown in Figure 5.4. The general feature for CDR3β length distribution appears to be a bell shape, with

sequences having 14-18 amino acid residues within CDR3β region are the dominant components and less sequences for CDR3β shorter than 14 amino acids or longer than 18 amino acids. This type of CDR3β length distribution, also known as Gaussian distribution, has been widely observed in both human and mouse studies [Miqueu et al., 2007]. More amino acid sequences were identified from sample 2 than from sample 1. CDR3β length distribution analysis in Figure 5.4 shows that the majority of these extra sequences in sample 2 had 15-18 amino acid residues within the CDR3β region.

Table 5-2 Summary of Illumina sequencing data of CDR3β transcripts with Vβ28-Jβ3.2/2.2 rearrangements from CD8 T cells derived from the PBMC of a previously *T. parva* immunized A10-homozygous animal (403957).

Sequencing reads, raw data	**1** 6,287,572/2 [a)]		**2** 7,579,198/2	
	High quality [b)] sequences	Unique CDR3β amino acid sequences	High quality sequences	Unique CDR3β amino acid sequences
Reads with valid Vβ and Jβ genes [c)]	2,934,553 (93%)	201,826	3,560,823 (94%)	287,097
CDR3β extraction [d)]	2,614,697 (83%)	144,211	2,981,400 (79%)	200,050
Reads with Vβ28 and Jβ3.2	2,051,452 (65%)	100,349	2,404,207 (63%)	132,170

[a)] For paired-end Illumina sequencing, half of the reads began from either 3'-end or 5'-end of the TRB sequence.

[b)] Total number of high-quality (≥Q30 quality score) nucleotide sequences. Percentages in parentheses are proportions of raw data used for analysis.

[c)] Sequencing reads with the defined Vβ28 and Jβ2.2/3.2 gene segments.

[d)] Identification of CDR3β region is according to the conserved amino acid Cys (C) located towards the 3' end of the V gene segment and the conserved amino acid Phe (F) in 5' end of the J gene segment.

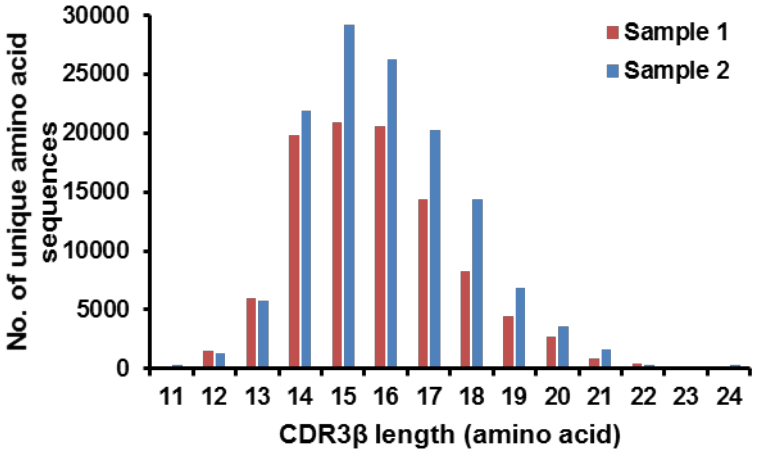


Figure 5.4 CDR3β length distributions for the unique amino acid sequences with Vβ28-Jβ3.2 rearrangements.

CDR3β lengths ranging from 11 to 24 amino acids were observed for both samples, with CDR3β lengths of 14-18 residues dominant.

5.3.2.2 Correction of errors in TRB sequencing

Due to the non-germline, hyper-variable CDR3β region, sequencing errors in HTS of antigen-specific repertoires is problematic and can lead to dramatic over-estimation of repertoire diversity. Although quantifying repertoire diversity was not a critical aim of the study it was considered necessary to obtain an estimation of the sequencing error rate to avoid mis-interpretation of the data – 2 alternative methodologies were employed.

The abundance of each identified Vβ28-Jβ3.2 amino acid sequence was examined. Figure 5.5 shows abundance distribution of sequences encoded by 1-30 sequencing reads. In both samples ~80% of the identified amino acid sequences (~80,000 and ~100,000 in samples 1 and 2 respectively) were represented by only a single read. This distribution of read frequency suggests that many of the apparently unique CDR3β amino acid sequences may have arisen as artifacts due to late PCR or sequencing errors. As shown in Figure 5.6, nucleotide sequences that encode these CDR3β amino acid sequences were calculated as 3.97% and 4.51% of the total sequencing reads with Vβ28-Jβ3.2 rearrangements in sample 1 and 2 respectively.

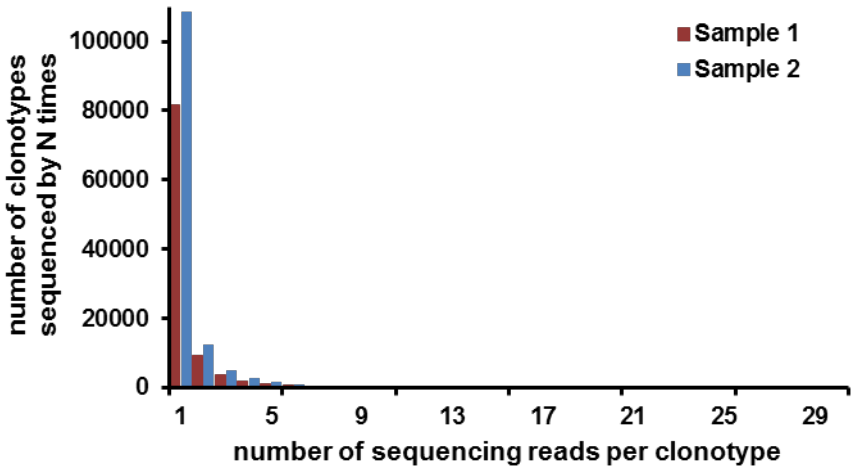


Figure 5.5 Abundance distribution of unique CDR3β amino acid sequences with 1-30 sequencing reads.

Over 80,000 and 100,000 CDR3β amino acid sequences were sequenced only once in the two datasets respectively.

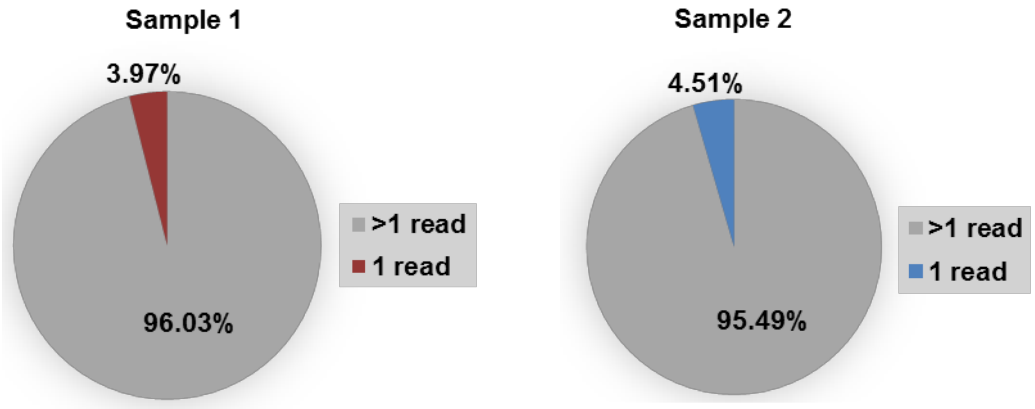


Figure 5.6 Percentages of nucleotide sequences encoding the unique CDR3β amino acid sequences with 1 read.

The encoding nucleotide sequences for the ~80% unique CDR3β amino acid sequences that were sequenced once in sample 1 and 2 are 3.97% and 4.51% of the total Vβ28-Jβ3.2 sequencing reads respectively.

The first error correction method used was based on an estimation of the error rate in the sequencing reads, similar to that described in [Warren et al., 2011]. Using the germline Vβ28 sequence, it was possible to determine that the error rate was 0.8766 bp/Kbp and 0.8876 bp/Kbp for sample 1 and 2 respectively (Table 5-3). As

the average length of the CDR3β region was ~50bp, an estimated 4.4% of the sequence reads are likely to contain a nucleotide error within the CDR3β. This calculated cutoff is very close to the percentages (3.97% and 4.51% in Figure 5.6) of single nucleotide sequences encoding unique CDR3β amino acid sequences in both datasets. Removing the 3.97% and 4.51% of single read sequences resulted in a diversity of ~2-3×10^4 unique CDR3β amino acid sequences.

Table 5-3 Error rates for sequencing data of sample 1 and 2.

	Erroneous nucleotides in Vβ	**Sequences analyzed**	**Vβ length (Kbp)**	**Error rate (errors/Kbp)**	**Average CDR3β length (Kbp)**	**Cutoff**
Sample 1	579,885	2,614,697	0.253	**0.8766**	0.05	**4.38%**
Sample 2	669,531	2,981,400	0.253	**0.8876**	0.05	**4.44%**

Error rates of sequence data for the two samples and the cutoff for low-abundant sequencing reads are calculated according to section 5.2.2. Error rate = total number of erroneous nucleotides in Vβ/ (total number of analyzed sequences × Vβ length). Cutoff = (error rate × CDR3β length × 100) %.

A more detailed error correction process (as described in section 5.2.2) has been described for HTS datasets of human and murine TCR [Bolotin et al., 2012]. This was applied to a representative subset of sequence data. In this process low-abundant reads with no more than a total of three mismatches in germline V, D and J gene components of the CDR3β region (in these regions mismatches between reads can only arise from PCR or sequencing errors) were mapped to a major 'core clonotype' sharing the same non-germline CDR3β sequence. By avoiding manipulation of the non-germline gene junctions, this correction step maintains the genuine CDR3β sequence diversity. A summary of this error correction is shown in Table 5-4 and the complete analysis is detailed in Appendix C. Notably some of the apparently erroneous sequences were represented by up to 25 reads, suggesting that some of the errors may have been introduced early during PCR (and thus subsequently amplified). For the example shown in Table 5-4, a total of 88 low-abundant clonotypes, encoding 49 unique CDR3β amino acid sequences, were corrected and mapped to the major clonotype, giving an indication of how PCR/sequencing error, if uncorrected,

can dramatically inflate the apparent CDR3β clonal diversity of samples analyzed by HTS.

During error correction, three types of low-abundant clonotypes (<25 sequencing reads in the above example) were observed; 1) clonotypes which differ only in germline V, D and/or J components that can thus be safely considered as erroneous and merged to dominant clonotypes - such as those shown in Table 5-4, 2) clonotypes with residue variants at gene junctions and 3) clonotypes that were removed due to the presence of more than three errors in the amplified V, D and J genes. Examples for the latter two types of clonotypes are shown in Table 5-5. Frequencies for the three types of low-abundant clonotypes for the sample analyzed were calculated as 70.9%, 22.3% and 6.8% respectively. The second type of clonotypes could be generated from non-germline nucleotide additions/deletions during the VDJ rearrangement. Therefore, these clonotypes could represent the real diversity of Vβ28-Jβ3.2 rearrangement. The proportion of clonotypes with less than 25 sequencing reads were found to encode up to 98% of the whole identified unique CDR3β amino acid sequences. Therefore, the realistic estimation of Vβ28-Jβ3.2 repertoire diversity is around 24% (2% + 98%×22.3%) of the sequences defined before error correction, which equals 2.4×10^4 for sample 1 and 3.2×10^4 for sample 2. However, the precise clonal diversity for Vβ28-Jβ3.2 rearrangement can only be verified after a comprehensive error correction for all identified sequencing reads. At the time the study was conducted, the lack of an efficient bioinformatics package for conducting error correction for bovine TCR data made it unfeasible to complete such an analysis.

Nevertheless, application of the two approaches to error correction (using a calculated cutoff and using an error correction algorithm based on sharing of non-germline CDR3β nucleotide sequence) provided very similar results – estimating that the realistic range of unique Vβ28/Jβ3.2$^+$ TRBs identified in each of the populations was $2\text{-}3\times10^4$.

Table 5-4 An example of error correction for merging low-abundant clonotypes with a dominant core clonotype.

Number of sequencing reads	CDR3, amino acid sequences	CDR3, nucleotide sequences	Vβ	Jβ	Dβ
3343	**CASDPSGGANTQPLYF**	**TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT**	**28**	**3.2**	**3**
25	CASDPSGGAN**A**QPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAAC**G**CCCAGCCCCTGTACTTT	28	3.2	3
21	CASDP**A**GGANTQPLYF	TGTGCCAGCGACCCT**G**CGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
17	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCC**G**TCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
10	CASDPSGGAN**P**QPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAAC**C**CCCAGCCCCTGTACTTT	28	3.2	3
6	CASDP**T**GGANTQPLYF	TGTGCCAGCGACCCT**A**CGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
5	CASDPSGGA**S**TQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGA**G**CACCCAGCCCCTGTACTTT	28	3.2	3
5	CASDPSGGA**I**TQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGA**T**CACCCAGCCCCTGTACTTT	28	3.2	3
5	CASDPSGGANTQPL**D**F	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTG**G**ACTTT	28	3.2	3
2	C**V**SDPSGGANTQPLYF	TGTG**T**CAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
2	CASDPS**V**GANTQPLYF	TGTGCCAGCGACCCTTCGG**T**GGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
2	CASDPSGGANTQPL**D**F	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTG**G**ACTTT	28	3.2	3
2	CASDP**A**GGAN**A**QPLYF	TGTGCCAGCGACCCT**G**CGGGGGGAGCGAAC**G**CCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGANTQ**L**L**S**F	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGC**T**CCTGT**C**CTTT	28	3.2	3
1	CASDPS**V**GA**T**TQPL**S**F	TGTGCCAGCGACCCTTCGG**T**GGGAGCGA**C**CACCCAGCCCCTGT**C**CTTT	28	3.2	3

The dominant "core clonotype", which determines the nucleotide sequence of the final clonotype, is shown in bold. Vβ (orange), Dβ (blue) and Jβ (green) gene components and corrected errors (red) within CDR3β nucleotide sequence are shown. A limited number of low-abundant clonotypes are shown. The complete table can be found in Appendix C.

Table 5-5 Examples of low-abundant clonotypes with residue variants at junctions and clonotypes with more than three errors within CDR3β

Number of sequencing reads	CDR3, amino acid sequences	CDR3, nucleotide sequences	Vβ	Jβ	Dβ
3343	**CASDPSGGANTQPLYF**	**TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT**	**28**	**3.2**	**3**
9	CASDRSGGANTQPLYF	TGTGCCAGCGACCGTTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
3	CASDPSGVANTQPLYF	TGTGCCAGCGACCCTTCGGGGGTAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
2	CASDPSGAANTQPLYF	TGTGCCAGCGACCCTTCGGGGGCAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
2	CASDHSGGANTQPLYF	TGTGCCAGCGACCATTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
2	CASDPSGGADTQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGGACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDLSGGANTQPLYF	TGTGCCAGCGACCTGTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPAVGANAQPLYF	TGTGCCAGCGACCCTGCGGTGGGAGCGAACGCCCAGCCGCTGTACTTT	28	3.2	3

The dominant "core clonotype", which determines the nucleotide sequence of the final clonotype, is shown in bold. V segments (orange), D segments (blue), J segments (green) and errors (red) within CDR3 nucleotide sequence are shown. Variations in sequences from the "core clonotypes" at gene junctions are shown in purple font and are underlined.

5.3.3 Assessment of coverage of the Vβ28-Jβ3.2 repertoire in the analyzed samples

To enable an evaluation of how comprehensively the Vβ28-Jβ3.2 repertoire in PBMC was being analysed using the HTS approach, the results from two aliquots of CD8 T cells derived from the same individual (403957) and having the same number of cells (1×10^7) were compared to identify how much commonality there was in the identified TRB repertoires. These two aliquots of CD8 T cells were isolated from the same batch of blood sample, processed at the same time, prepared using identical procedures for mRNA isolation, cDNA synthesis, PCR amplification and purification, and the same quantity of purified PCR product was sent for sequencing.

As can be seen in Table 5-2, more sequencing reads were obtained from sample 2 and there was a commensurate greater number of unique amino acid sequences encoded by the Vβ28-Jβ3.2 rearrangements in sample 2 compared to sample 1 (132,170 vs. 100,349) in the uncorrected datasets. Notably, only 16,194 CDR3β amino acid sequences were found in both datasets (Figure 5.7A); this is ~16% and 12% of the amino acid sequences identified in samples 1 and 2 respectively. These values are based on the uncorrected sequence data and so may under-estimate the true degree of sequence overlap between the 2 samples. Sequence overlap was then analyzed for the two samples after removing the 3.97% and 4.51% of single read sequences. As shown in Figure 5.7B, from $2\text{-}3\times10^4$ CDR3β amino acid sequences, only 6,426 sequences were shared by the two datasets, which is 30% and 22.5% of the amino acid sequences in samples 1 and 2 respectively after a crude cutoff.

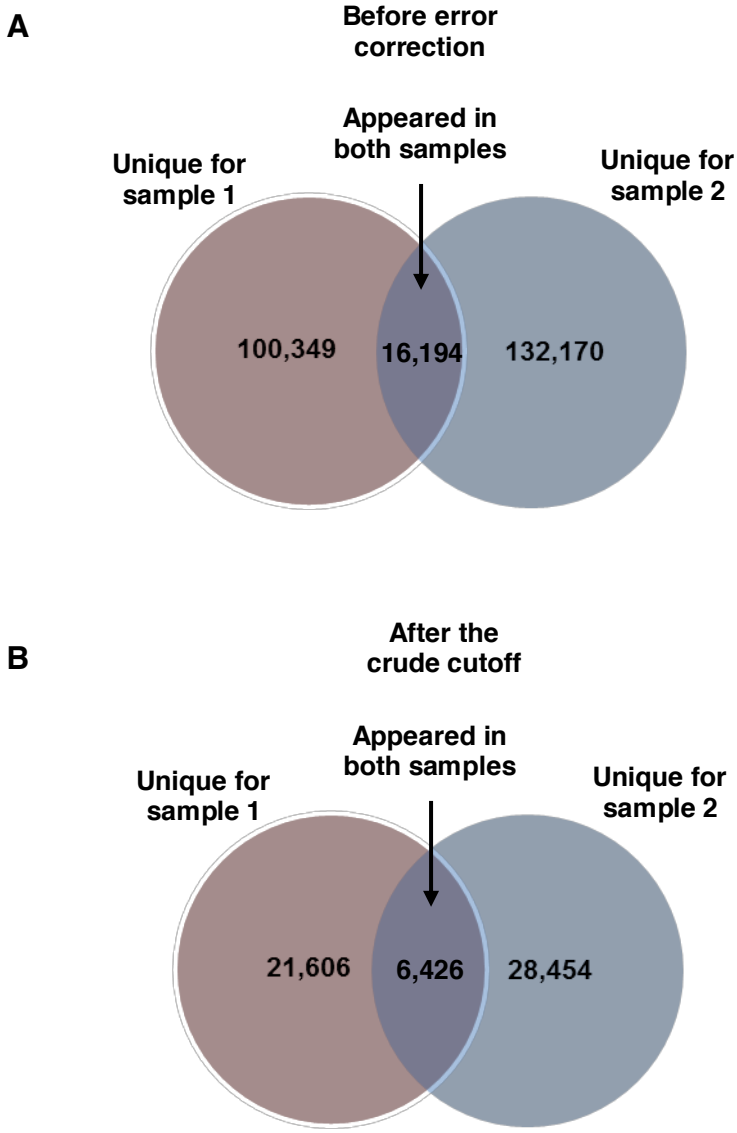


Figure 5.7 Proportion of Vβ28-Jβ3.2 amino acid sequences shared between samples 1 and 2.
A, from identified unique CDR3β amino acid sequences with Vβ28-Jβ3.2 rearrangements, 16,194 sequences were found in both samples, 16.1% of amino acid sequences for sample 1 and 12.2% of amino acid sequences for sample 2. B, after the cut-off of the 3.97% and 4.51% of single read sequences, 2-3×10^4 unique CDR3β amino acid sequences were obtained for samples 1 and 2. Within these sequences, 6,426 amino acid sequences were found in both samples, 30% of amino acid sequences for sample 1 and 22.5% of amino acid sequences for sample 2.

5.3.4 Identification of potential epitope-specific TRB rearrangements

Results from the previous chapter have demonstrated the presence of the conserved TRB sequence Vβ28-CASAEYGGENTQPLYF-Jβ3.2 in the $Tp2_{50-59}$ epitope-specific CD8 T cells from animal 403957 (6% of *in vitro* generated CD8 T cell line, Figure 3.1). However, the exact sequence of the conserved Vβ28-CASAEYGGENTQPLYF-Jβ3.2 rearrangement was not found within the diverse Vβ28-Jβ3.2 rearrangements obtained in either sample 1 or 2. Instead, sequences that encode 1 amino acid substitution from the conserved TRB sequence were observed. As shown in Table 5-6, errors (in red font) within germline V, D and J gene components of CDR3β were corrected using the method as above and six unique CDR3β amino acid sequences (1 high- and 5 low-abundant sequencing reads) were identified. The one residue (in purple font and underlined) differing between these sequences and the expected TRB sequence (Vβ28-CASAEYGGENTQPLYF-Jβ3.2) was located at the non-germline Dβ-Jβ gene junction. Of the six unique CDR3β amino acid sequences, only one sequence was identified from both samples, but encoded by significantly different number of sequencing reads (1267 reads from sample 1 and 1 read from sample 2). Four sequences only appeared in sample 1 and one sequence only appeared in sample 2. These results also suggest that sequencing reads obtained from each of the two samples are likely not sufficient to cover all potential Vβ28-Jβ3.2 rearrangements.

Interestingly, the Vβ28-CASAEYGGKNTQPLYF-Jβ3.2 clonotype has been identified in the $Tp2_{50-59}$ epitope-specific CD8 T cells obtained from cattle 302186 in the previous chapter (Table 4-3), suggesting that this TRB (and possibly others that have an amino acid substitution in this position) is also expressed by $Tp2_{50-59}$-specific CD8 T cells.

Table 5-6 Sequencing reads with one amino acid variant comparing with the defined TRB clonotypes Vβ28-CASAEYGGENTQPLYF-Jβ3.2.

Number of sequencing reads Sample 1	Sample 2	CDR3, amino acid sequence	CDR3, nucleotide sequence	Vβ	Jβ	Dβ
Conserved		***CASAEYGGENTQPLYF***	***TGTGCCAGC**GCTGAATA**TGGGGGG**GA**GAACACCCAGCCCCTGTACTTT***	***28***	***3.2***	***2***
1245	1	**CASAEYGGTNTQPLYF**	TGTGCCAGCGCTGAATACGGGGGGACGAACACCCAGCCCCTGTACTTT	28	3.2	3
2		CASAEYGGTNTQPLYF	TGTGCCAGCGCTGAATACGG**T**GGGACGAACACCCAGCCCCTGTACTTT	28	3.2	3
10		CASAEYGGTNTQPLYF	TGTGCCAGCGCTGAATACGG**C**GGGACGAACACCCAGCCCCTGTACTTT	28	3.2	3
2		CASAEYGGTNTQPLYF	TGTGCCAGCGCTGAATACGG**C**GGGACGAACACCCAGCC**G**CTGTACTTT	28	3.2	3
2		CASAEYGGTNTQPLYF	TGTGCCAGCGCCGAATACGG**C**GGGACGAACACCCAGCCCCTGTACTTT	28	3.2	3
1		CASAEYGGTNTQPLYF	TGTGCCAGCGCTGAATACGG**C**GGGACGAACAC**G**CAGCCCCTGTACTTT	28	3.2	3
2		CASAEYGGTNTQPLYF	TGTGCCAGCGCTGAATACGG**C**GGGACGAACACCCAGCCCCT**T**TACTTT	28	3.2	3
2		CASAEYGGTNTQPLYF	TGTGCCAGCGCTGAATACGG**C**GGGACGAACACCCAGCCCCT**A**TACTTT	28	3.2	3
1		**CASAEYGGANTQPLYF**	TGTGCCAGCGCTGAATACGGGGGGGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
5		CASAEYGGANTQPLYF	TGTGCCAGCGCTGAATACGG**C**GGGGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1		CASAEYGGANTQPLYF	TGTGCCAGCGCTGAATACGGGGGGGC**A**AACACCCAGCCCCTGTACTTT	28	3.2	3
1		**CASAEYGGVNTQPLYF**	TGTGCCAGCGCTGAATACGGGGGAGTGAACACCCAGCCCCTGTACTTT	28	3.2	3
1		CASAEYGGVNTQPLYF	TGTGCCAGCGCTGAGTACGGGGGTGTGAACACCCAGCCCCTGTACTTT	28	3.2	3
1		***CASAEYGGKNTQPLYF***	*TGTGCCAGC**GCTGAATA**CGG**AGGG**AA**GAA**TACCCAGCCCCTGTACTTT*	*28*	*3.2*	*3*
1		**CASAEYGGMNTQPLYF**	TGTGCCAGCGCTGAGTACGGGGGTATGAACACCCAGCCCCTGTACTTT	28	3.2	3
	4	**CASAEYGGGNTQPLYF**	TGTGCCAGCGCTGAGTATGG**A**GGGGGGAACACCCAGCCCCTGTACTTT	28	3.2	3

Nucleotide and amino acid sequences for the Vβ28-Jβ3.2 rearrangements with one residue variation compared to the conserved Vβ28-CASAEYGGENTQPLYF-Jβ3.2 rearrangement are shown. Number of sequencing reads for each amino acid sequence is shown for both samples. Germline V, D and J gene components of CDR3β are shown in orange, blue and green fond respectively. Nucleotide mismatches in germline gene components are in red. Amino acid variants in non-germline Dβ-Jβ junctions are in purple and underlined. Unique amino acid sequences are shaded.

5.4 Discussion

In order to investigate the potential influence of CD8 T cell repertoires to Tp2 epitope dominance variation between A10-homozygous and -heterozygous cattle, work in this chapter aimed to establish a system enabling the detection of epitope-specific CD8 T cells directly *ex vivo*. In the previous chapter, TCRβ repertoires of epitope-specific CD8 T cells were analysed and a conserved TCRβ clonotype with Vβ28-CASAEYGGENTQPLYF-Jβ3.2 rearrangement was identified to be shared by the $Tp2_{50-59}$ epitope-specific CD8 T cells from 5 A10-homozygous cattle. In the current work, this specific TRB clonotype was used as a 'barcode' to serve as a proxy for the detection of $Tp2_{50-59}$-specific CD8 T cells.

The application of high throughput sequencing (HTS) technology for analysing immune repertoires such as TCR provides a high resolution picture of repertoire usages for a complex CD8 T cell population [Calis and Rosenberg, 2014, Six et al., 2013]. Initial experiments were conducted to provide 'a proof of concept' that HTS could be used to identify the 'public' TCRβ chain directly *ex vivo*. To achieve this, purified CD8 T-cells from the PBMC of a *T. parva*-immunised A10-homozygous animal, from which the 'public' TCRβ chain had been identified from *in vitro* cultured CD8 T cells previously, were analysed. From the resultant sequencing data, the public TRB clonotype was not detected. As the current study was to validate the utilization of HTS for detecting epitope-specific TRB clonotype, the result suggests that further optimisation of the methodology is required before continuing the analyses of naïve CD8 T cell repertoires in A10-homozygous and –heterozygous cattle.

High quality sequencing reads were obtained, indicating the success of the sequencing approach at the technical level. However, TCR repertoire analysis using HTS is generally challenging due to 1) the highly diverse repertoires being analysed [Arstila et al., 1999] and 2) the presence of hypervariable, non-germline sequences incorporated during the process of somatic recombination [Schatz and Ji, 2011] which means that differentiation of error and genuine sequence variation is difficult. Although error correction was not a priority of this study, a realistic estimation of the

diversity of Vβ28-Jβ3.2 rearrangements would allow the evaluation of repertoire coverage in the analysed samples, since identification of the public TRB rearrangement Vβ28-AEYGGENTQPLYF-Jβ3.2 requires a sufficient sequencing depth for the Vβ28-Jβ3.2 repertoires.

Error correction for TRB sequences is widely recognized as difficult due to base pair changes in the non-germline coding region potentially being a result of error or genuine sequence variants between clonotypes. Erroneous clonotypes could hugely inflate the clonotypic diversity in analysed samples. Studies using human and mouse models have shown that many of the aberrant clonotypes are present at low frequency, suggesting that errors are introduced either later in PCR or during sequencing [Shugay et al., 2014, Bolotin et al., 2012, Nguyen et al., 2011]. Various approaches have been employed for error correction. One method was to remove sequences with low abundant reads based on a calculated error rate [Warren et al., 2011]. A 4.4% cutoff was applied in the current study based on an error rate of 0.88 erroneous nucleotides per 1000bp sequencing reads (Table 5-3). Removing a corresponding proportion of low-abundant sequencing reads resulted in 80% reduction of Vβ28-Jβ3.2 amino acid sequence diversity. However, this approach could be problematic due to removing of genuine clonotype variants, and result in an underestimation of real repertoire coverage by the sequencing data. A more sophisticated approach [Bolotin et al., 2012] was then used to remove only PCR/sequencing artefacts by targeting germline V, D and J sequences for error correction. However, an efficient bioinformatics package [Bolotin et al., 2013] is required for this method, which is currently not available for bovine TCR (although these are being developed - private communication to Dr. Connelley). To provide an indication of how such correction would affect the data generated in this study, manual correction was performed on a representative clonotype (Table 5-4 and Table 5-5). Based on the analysis of this one sample, a similar level of Vβ28-Jβ2.3 repertoire diversity ($2.5\text{-}3.2 \times 10^5$) was estimated as the previous approach applying a crude cutoff.

To generate sequencing data, 1×10^7 CD8 T cells were used, which is equivalent to the numbers of CD8 T cells used for establishing the *T. parva*-specific CD8 T cell

cultures from which the public TRB sequence was identified. Therefore, the assumption was that the samples used were sufficiently large to permit the identification of the public Vβ28-Jβ3.2 TRB rearrangement. Previous studies have identified ~90 functional Vβ and 17 Jβ germline genes [Connelley et al., 2009]. Assuming that the use of these gene segments is evenly distributed during TRB rearrangement (which is unlikely to be the genuine *in vivo* situation but allows an approximate calculation to be made), it is possible to estimate that within 1×10^7 CD8 T cells, 1.3×10^4 ($1\times10^7 \times 1/90 \times 2/17$) cells will express TRB chains with a Vβ28-Jβ2.2/3.2 rearrangement. The sequencing depth achieved in samples 1 and 2 (2×10^6 and 2.4×10^6 high-quality sequences respectively) therefore provided more than 100-fold sequencing coverage of the estimated repertoire. In fact, the number of Vβ28-Jβ3.2 TRB amino acid sequences obtained (ranging from 1-1.3×10^5 before and 2-3×10^4 after error correction) was greater than that estimated. However, comparison of TRB repertoires of the two samples (derived from the same animal and processed at the same time) revealed limited overlap of the Vβ28-Jβ3.2 TRB amino acid sequences as shown in Figure 5.7. A similar level of TRB amino acid sequence sharing has been reported in human models, although TRB sequences were derived from different individuals in the study [Warren et al., 2011]. This limited proportion of shared Vβ28-Jβ3.2 amino acid sequences indicates that the data obtained in the current study may not be sufficient to exhaustively sequence all of the Vβ28-Jβ3.2 repertoire in the analysed memory CD8 T cell populations and this, rather than insufficient biological sampling may account for the failure to identify the targeted public TCR.

A review of the current literature suggests that use of HTS to track antigen-specific CD8 T cell clonotypes in unsorted memory populations has not been attempted previously. In a study of human CD8 T cell repertoires against HCMV, antigen-specific clonotypes were tracked in memory populations [Klarenbeek et al., 2012]. However, sequencing data obtained in that study were from pMHC I tetramer sorted CD8 T cells and furthermore in HCMV antigen-specific CD8 T cell clonotypes are known to continuously expand over a period of time. Studies conducted in this project, instead, attempted to track a single epitope-specific TRB

sequence in the whole memory population. As this TRB may be present at low frequency during a quiescent memory stage, the ability to effectively and comprehensively cover the full TRB repertoire in sequenced samples may be of critical importance. The results presented in this chapter suggest that problems still exist in the application of HTS to detect antigen-specific T cell clonotypes in unsorted memory or naïve CD8 T cell populations.

Chapter 6: Transduction of bovine CD8 T cells for the expression of epitope-specific TCR

6.1 Introduction

Studies carried out in humans and mice have clearly demonstrated that naive CD8 T cell populations go through three stages of development *in vivo* during responses to infection or vaccination, namely expansion, contraction and differentiation, leading to the establishment of a memory CD8 T cell population [Kaech et al., 2002]. The frequency of CD8 T cells specific for individual epitopes in naïve T cell populations is extremely low. For example, it was estimated that a mouse possesses between 10 and 1000 naïve CD8 T cells capable of responding to a specific antigen [Blattman et al., 2002, Bousso et al., 1998, Casrouge et al., 2000]. This small population is activated after encountering antigen presenting cells (APCs), usually dendritic cells (DCs), and then undergo robust proliferation and differentiation to become effector T cells. A proportion of the activated CD8 T cells acquire cytolytic activity and are known as cytotoxic T lymphocytes (CTLs). Thus, fully activated CD8 T cells can kill infected cells or release cytokines to inhibit pathogen replication and survival [Heath and Carbone, 2001b, Harty et al., 2000]. The majority (90 - 95%) of effector cells die following the pathogen clearance and surviving cells differentiate into memory cells, a heterogeneous population characterized by the ability to react rapidly against subsequent challenge with the same pathogen [Miller et al., 2008, Callan et al., 2000]. There is evidence that the functional capacity and homeostasis of memory CD8 T cell populations strongly influence the level of immune protection [Seder et al., 2008, Farber et al., 2014].

For efficient activation of a naïve antigen-specific CD8 T cell, three necessary signals (commonly referred to as Signals 1, 2, 3) are required [Kaech and Cui, 2012]. Appropriate antigen stimulation via the T cell receptor (TCR) following recognition of antigenic peptide presented on MHC I molecules provides the initial activation signal (signal 1). The overall strength of this signal can be influenced by both the amount of available antigen and TCR affinity for the peptide-MHC, and determines the function and differentiation of antigen-specific CD8 T cells [Zehn et al., 2012].

However, without additional co-stimulation, recognition of antigen alone can result in anergy or deletion of the responding naïve CD8 T cells [Mescher et al., 2006]. Therefore, activation signal (signal 2) involving the engagement of co-stimulatory molecules such as CD28 and CD40 ligand on CD8 T cells with CD80 and CD40 on APCs is essential for productive CD8 T cell activation [Acuto and Michel, 2003]. Pro-inflammatory cytokines such as IL-12 and type I interferon (IFNα and β), representing signal 3, also appear to be critical, especially for the formation of memory CD8 T cell populations [Harty and Badovinac, 2008]. The particular cytokines that provide signal 3 depend on the individual pathogen and the pattern recognition receptor (PRR) signalling pathways it activates in APCs [Thompson et al., 2006, Haring et al., 2006]. In addition to these signals, there is increasing evidence for the requirement of CD4 T cells during the generation of optimal CD8 T cell effector or memory responses against pathogenic infections [Bevan, 2004]. The precise function and mechanisms of CD4 T cell involvement are still unclear. There is strong evidence that CD4 T cells are essential for survival and maintenance of effector and memory CD8 T cells following activation [Novy et al., 2007, Janssen et al., 2005, Sun et al., 2004]. Other findings suggest that CD4 T cell help is required for programming the induction of CD8 T cell responses [Crispe, 2014, Smith et al., 2004].

The protective function of bovine CD8 T cell responses against *T. parva* infection has been demonstrated by adoptive transfer of CD8 T cell-enriched populations between immune and naïve twin calves [McKeever et al., 1994, Morrison et al., 1987]. However, a study of responses to primary infection of calves with a lethal dose of *T. parva* sporozoites demonstrated that, although infection provoked a potent CD8 T cell response, the responding cells did not show parasite-specific cytolytic activity and did not appear to exert any control of the infection [Houston et al., 2008]. It was proposed that there was a defect in the functional differentiation of the responding T cells. Experiments in which defined *T. parva* antigens recognized by bovine CD8 T cells were used to immunize cattle by prime-boost protocols using plasmid DNA and poxvirus vectors, have also indicated that the functional capacity of the CD8 T cell response may be important [Graham et al.,

2006]. Immunisation resulted in antigen-specific CD8 T cell responses in most of the animals; however, in most animals the CD8 T cells did not show cytotoxic activity [Graham et al., 2006]. Again, it was suggested that naïve antigen-specific CD8 T cells may not have undergone appropriate functional differentiation. Although great advances have been made for understanding the role of CD8 T cell mediated immune responses in immunity against *T. parva* [Morrison, 2009], limited information is available on the factors that influence the effective induction of a protective CD8 T cell responses. *In vitro* experiments, involving mixing of different combinations of purified CD4 and CD8 T cell populations from *T. parva*-immune and naïve MHC-identical animals, suggested that immune CD4 T cells are required for efficient recall of a parasite-specific CD8 T cell response [Taracha et al., 1997]. Further investigation would be informative for a better understanding of the role of CD4 T cells in the generation and recall of memory CD8 T cell responses against *T. parva*.

A major obstacle to tracking the development of antigen-specific CD8 T cell responses in naïve animals is the extremely low frequency of antigen-specific cells in circulating CD8 T cell populations [Blattman et al., 2002, Bembridge et al., 1995]. In mice, generation of transgenic lines of animals expressing T cell receptors of defined epitope specificity has been used to overcome this obstacle. Studies of these TCR transgenic mice have provided valuable insights into induction of T cell responses in different disease models [Chen and Zavala, 2013, Li et al., 2010, Bumann, 2003]. However, this transgenic approach is not currently feasible for studies in large outbred species such as cattle. MHC I tetramer staining combined with magnetic beads-based cell enrichment has been used to purify naïve antigen-specific CD8 T cells from mouse models [Obar et al., 2008]. However, only very small numbers of cells can be obtained and even low levels of unspecific staining can result in low cell purity when staining cell populations containing rare positive cells. An alternative approach involving generation of TCR-transduced CD8 T cells *in vitro* has been used in mice and humans [Morgan et al., 2006, Kessels et al., 2001]. Adequate number of antigen-specific T cells can be generated from naïve CD8 T cells transduced *in vitro* with a TCR of defined antigen specificity; moreover, adoptive transfer of these CD8 T cells into laboratory animals has facilitated studies of effective memory CD8 T cell

response development [Kearney et al., 1994, Stemberger et al., 2007, Harty and Badovinac, 2008]. The current study aimed to establish some of the tools and reagents to enable this approach to be pursued to investigate responses of naïve antigen-specific CD8 T cells in cattle.

The first requirement to undertake TCR gene transfer is to identify paired functional TCR α and β chains expressed by T cells of the desired antigen specificity. To date, studies of TCR gene expression by *T. parva*-specific CD8 T cells have mainly focused on the TCRβ chain repertoires and little is known about TCRα chain usage. Rearrangement of the TCRα chain occurs after TCRβ chain rearrangement during cell development in the thymus; thus, two developing T cells with the same TCRβ chain rearrangement may have different rearranged TCRα chains. Moreover, unlike TCRβ chain, mature T cells frequently express two recombined TCRα chains [Gascoigne and Alam, 1999, Han et al., 2014]. This is due to the absence of genomic allelic exclusion and therefore generation of rearrangements of TCRα chains from both TCR haplotypes [Gascoigne and Alam, 1999]. When two apparently functional rearrangements occur, only one of the TCRα chains is believed to engage in antigen recognition, although both TCRα chains can be expressed on the cell surface [Ni et al., 2014, Heath and Miller, 1993]. For TCR gene transfer, the second requirement is an efficient transgene delivery system. A lentiviral vector was used for its ability to efficiently transduce non-dividing T cells, although activation signals are still required for efficient transduction [Unutmaz et al., 1999].

From a panel of Tp2 epitope-specific CD8 T cell clones, paired TCR α chain expressions for a defined TCR β chain were analysed. Epitope specificity for one of the identified TCRαβ pairs was confirmed to have functional activity, which provides a fundamental material for future generation of bovine naïve antigen-specific CD8 T cells. Experiments conducted in this chapter also attempted to validate the efficiency of lentiviral transduction of bovine CD8 T cells. Preliminary data of successful transgene expression on activated bovine CD8 T cells provides promising evidence for future utilization of lentivirus constructs to transduce bovine T cells.

6.2 Materials and methods

6.2.1 Generation of lentiviral constructs

Details of the methods used to obtain lentiviral-bovine TCR constructs are provided in section 2.3.4. Briefly, full-length cDNAs of paired TCR α and β chain transcripts were amplified from CD8 T cell clones of defined epitope specificity, and then combined by linking with a T2A 'self-cleaving' sequence in order to express two single chains driven by one promoter. The combined sequence was cloned into a lentiviral vector pSxW.tCD34. Plasmids with the generated lentiviral-bovine TCR constructs were obtained for preparing pseudotyped lentivirus particles.

6.2.2 Preparation of pseudotyped lentivirus particles

Details of pseudotyped lentivirus generation are provided in section 2.2.12. Briefly, 293 T cells were transfected with the generated lentiviral-TCR vectors along with the packaging vectors pCMVΔ8.91 and pMD2.G, using $CaCl_2$ transfection method. Viral particles collected from supernatant of cell cultures were concentrated by ultracentrifugation and stored at -80°C.

6.2.3 Virus titer

The viral titer here refers to the concentration of transducing units. Virus titer was determined by transduction of the TCR^- human leukemia Jurkat reporter cell line JRT3-T3.5 using serially diluted viral suspensions. To start with, 200µl of freshly prepared or stored virus stock were mixed with 1800µl of standard cell culture media. This virus suspension was diluted further to the desired concentration and 1ml added to 1×10^6 seeded Jurkat cells in 1 well of a 24-well plate, as described in section 2.2.13. The percentage of cells expressing the transgene was determined by immunofluorescence staining of a human CD34 tag gene expression and flow cytometry 72 hours post transduction. Since the vector number is generally higher than one at >30% of transduction, the titer was determined from the dilution that generated <30% of transgene-expressing cells according to the formula indicated below [Swainson et al., 2008, Kustikova et al., 2003].

$$\text{Titer (transduction units/ml)} = \frac{(\%\ \text{transgene}^{+}\ \text{cells}/100) \times \text{number of cells per well}}{\text{total volume of virus (ml)}}$$

6.2.4 Luciferase assay

The reporter cell line JRT3-T3.5 used here has been transfected with a luciferase reporter construct under the transcriptional control of nuclear factor of activated T cells (NFAT), a transcription factor that is induced upon TCR signaling [Karttunen and Shastri, 1991]. For activation, 10^5 Jurkat cells transduced with lentivirus were stimulated for 16 hours with 1.5-2 × 10^4 peptide loaded antigen presenting cells (592 TA, see section 3.2.5 for peptide loading). The supernatant was then collected for luciferase detection using BioLux® Gaussia Luciferase Assay Kit (New England Biolab) according to the manufacturer's instructions. The maximal activity of the luciferase reporter construct was determined by stimulation of the Jurkat reporter cells with 5ng/ml PMA and 1 µM ionomycin for 4 hours. Non-stimulated reporter cells were set up as negative control. The luciferase activity of stimulated transduced Jurkat cells was expressed in relative luminescence activity, calculated according to the formula RLU (%) = (Release from tests -minimum release) x 100/ (Maximum release -minimum release) [Aarnoudse et al., 2002].

6.2.5 Pre-activation of bovine CD8 T cells

To facilitate lentiviral transduction, anti-CD3 and anti-CD28 monoclonal antibodies, which provide strong activation and proliferation signals through the T cell receptor complex, were used to activate bovine CD8 T cells. One day before cell stimulation, the anti-bovine CD3 antibody (MM1A) was added to the wells of a 96-well flat-bottom plate (not-treated, Costar) at a final concentration of 1µg/ml in PBS and incubated at 4°C overnight.

On the day of stimulation, unbound antibody was removed from the wells and purified anti-bovine CD28 antibody (low endotoxin, AbD Serotec) and recombinant rIL2 were added along with 5×10^4 T cells/well. The volume was adjusted to 200µl per well, giving a final concentration of 5µg/ml anti-CD28 and 100U/ml of rIL2. The plates were incubated at 37°C in 5% CO_2 for 24 hours. To confirm cell activation of

the CD8 T cells, preliminary experiments were carried out to measure activation at 20 hours after stimulation, using cell cycle analysis. Cell cycle staining was performed using Hoechst 33285 dye (Sigma). Briefly, 10µl of Hoechst 33285 dye (at a concentration of 1mM) were added to cells maintained in the culture medium and incubated for 30min at 37°C. The cells were analysed by flow cytometry as soon as possible without washing out the stain. FlowJo software was used for cell cycle analysis.

6.2.6 Lentiviral transduction of bovine CD8 T cells

Details of lentiviral transduction of bovine CD8 T cells are presented in section 2.2.14. Briefly, primary bovine CD8 T cells were purified from PBMC by MACS sorting and then pre-stimulated with anti-bovine CD3/CD28 antibodies for 24 hours in the presence of rIL2, to achieve activation. For virus transduction, the viral particles were added to the pre-activated bovine CD8 T cells at a multiplicity of infection (MOI) of 1~10. Polybrene was added to a final concentration of 6µg/ml to increase the efficiency of transduction.

6.3 Results

6.3.1 Identification of paired TCR α and β chains expressed by CD8 T cell clones

In the previous chapter, analyses of the TCR repertoires of epitope-specific CD8 T cells focused on the expressed TCRβ chains. Hence, information on the α chains expressed along with the β chains in individual T cells was not available. To determine the paired TCRα chain usages in CD8 T cells specific for the defined $Tp2_{50-59}$ epitope, CD8 T cell clones specific for this epitope were generated (according to section 2.2.6). The experiments focused on a TCRβ clonotype (Vβ28-CASAEYGGENTQPLYF-Jβ3.2), for which studies described in the previous chapter showed was conserved in the CD8 T cell responses of three A10-homozyous cattle. This clonotype had also been reported in two additional A10-homozygous cattle in a previous study [Connelley et al., 2011]. The TCRα chain usage of the $Tp2_{50-59}$ epitope-specific CD8 T cell clones expressing this conserved TCRβ chain was investigated in four of the A10 homozygous cattle. A PCR employing primers specific for Vβ28 and Jβ3.2 gene segments (as described in section 4.2.3) was first used to identify T cell clones expressing these gene segments. As shown in Figure 6.1, clones yielding positive PCR results were identified in all 4 animals. PCR products from positively amplified clones were then sequenced to confirm the presence of the specific Vβ28-CASAEYGGENTQPLYF-Jβ3.2 TCRβ chain rearrangement. Sequencing results determined that a total of 37 CD8 T cell clones were generated with the defined TCRβ chain expression, as listed in Table 6-1. Of these 37 CD8 T cell clones, 21 were used for TCRα chain amplification. The numbers of clones used from each animal are also shown in Table 6-1.

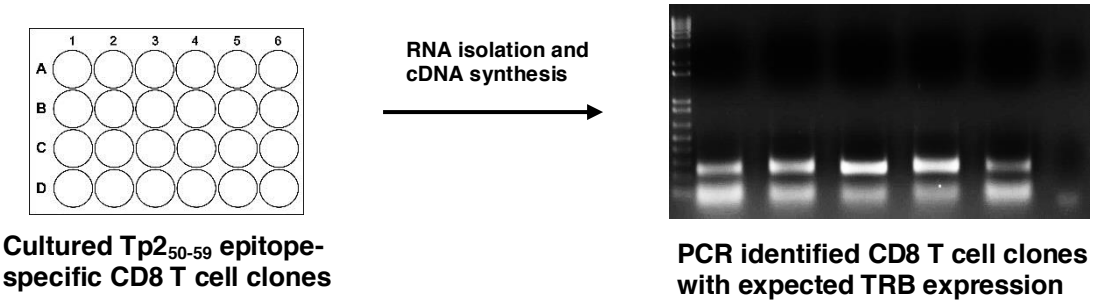


Figure 6.1 Selection of Tp2$_{50-59}$ epitope-specific CD8 T cell clones with Vβ28-CASAEYGGENTQPLYF-Jβ3.2 rearrangement.

cDNAs of generated Tp2$_{50-59}$ epitope-specific CD8 T cell clones were used for PCR identification of clones with the defined TRB expression.

Table 6-1 Number of Tp2$_{50-59}$ epitope-specific CD8 T cell clones with the defined TRB rearrangement from each A10 homozygous cattle

Animal	Total number of CD8 T cell clones	Number of clones with the defined TRB	Number of clones used for TCRα amplification
302186	46	9	5
403992	17	4	3
403957	20	4	3
1011	-	20	10

Number of CD8 T cell clones with the defined TCR β chain expression and total number of CD8 T cell clones from which the defined clones were selected are shown for the respective A10 homozygous cattle. CD8 T cell clones were generated from the 3 A10-homozygous cattle (302186, 403992, 403957) used in this study. Previously generated CD8 T cell clones from animal 1011 were also used. -, number unavailable. Number of clones used for TCRα expression analysis is also shown for each animal.

Using PCRs employing a panel of forward primers that amplify groups of known bovine Vα gene subfamilies and a reverse primer specific for the bovine Cα gene as described in section 2.3.4.1, TCRα chain transcripts were amplified from 21 of the Tp2$_{50-59}$ epitope-specific CD8 T cell clones. Single-band PCR products were obtained from these clones, for one or more of the Vα gene-specific primers. Summaries of TCRα chain analysis for these clones are shown in Table 6-2. PCR

products from 12 of the 21 CD8 T cell clones were sequenced to identify in-frame TCRα chain sequences. For 11 clones, one in-frame TCRα chain was identified for each clone, while for one clone from animal 302186, two in-frame TCRα chains were identified.

Table 6-2 Summary of TCRα chain analysis for CD8 T cell clones from A10-homozygous cattle.

Animal	Number of clones				
	PCR products for Vα primers		Sequenced clones	In-frame TCRα chains	
	One primer	Multiple primers		One	Two
302186		5	1		1
403992		3	2	2	
403957		3	1	1	
1011	10		8	8	

Numbers of the $Tp2_{50-59}$ epitope-specific CD8 T cell clones used for TCRα chain amplification and sequencing are shown for each of the four A10-homozygous cattle. Numbers of clones with one or two identified in-frame TCRα chains are also shown for each individual.

Sequencing of the PCR products of those clones that yielded more than two PCR products indicated that this was due to cross-reactivity of the PCR primers. Figure 6.2 shows representative PCR amplification results for 4 CD8 T cell clones from 4 animals. Three clear single PCR bands were amplified from CD8 T cell clone 2186.31 with primers designed to be specific for Vα8.4, Vα9 and Vα13 gene subgroup respectively (Figure 6.2A). The same result was obtained for all 5 clones derived from the same animal. The Sequence data from this clone showed two in-frame TCRα rearrangements, with Vα8.4 and Vα13 gene segments. The sequence amplified using the Vα9-specific primer was identical to that obtained using the Vα13-specific primer, both having the same Vα13-Jα20 rearrangement (Table 6-3). PCR products amplified using Vα9 and Vα13 specific primers were also obtained for the 3 CD8 T cell clones from animal 403957, as shown for clone 3957.11 in Figure 6.2B. Sequencing results of the two PCR products from clone 3957.11 showed the same Vα13-Jα49 rearrangement (Table 6-3). These results demonstrate that under the PCR conditions used there is cross-reactivity of Vα9-specific primer with Vα13.

PCR products amplified with multiple Vα gene-specific primers were also observed for 3 CD8 T cell clones from animal 403992. Two patterns of PCR

amplification were observed, as shown for clone 3992.12 and 3992.8 in Figure 6.2C and Figure 6.2D respectively. Sequencing of PCR products from clone 3992.12 showed only one in-frame TCRα chain rearrangement Vα12-Jα34 (Table 6-3); PCR products amplified using Vα8.1 and Vα20 subgroup-specific primers are out-of-frame. For the clone 3992.8, PCR products amplified using Vα4 and Vα26 subgroup-specific primers showed the same in-frame TCRα rearrangement Vα26-Jα22, suggesting the cross-reactivity of Vα4-specific primer with Vα26 under the PCR conditions. PCR products amplified using Vα8.1 and Vα29 subgroup-specific primers are out-of-frame. Therefore, for the two CD8 T cell clones from animal 403992, one in-frame TCRα chains was identified for each clone.

For animal 1011, TCRα transcripts were amplified from 10 CD8 T cell clones and all of them had only one PCR product, amplified using the Vα26 subgroup-specific primer as shown for clone 1011.35 in Figure 6.2E. PCR products of 8 clones were sequenced and shown to have the same in-frame TCRα rearrangement Vα26-Jα8.2.

In total, two in-frame TCRα chains were identified from 1 clone of animal 302186; two in-frame TCRα chains were identified from 2 clones of animal 403992; one in-frame TCRα chain was identified from 1 clone of animal 403957 and one in-frame TCRα chain was identified from 8 clones of animal 1011. Sequences of all the identified TCRα rearrangements are shown in Table 6-3. CD8 T cell clones from each individual showed unique TCRα chain usage, although they all have the same TCRβ chain. The observation that two different TCRα chains were used by two CD8 T cell clones (3992.12 and 3992.8) from the same individual (403992) suggests they arose from different clones in the naïve repertoire.

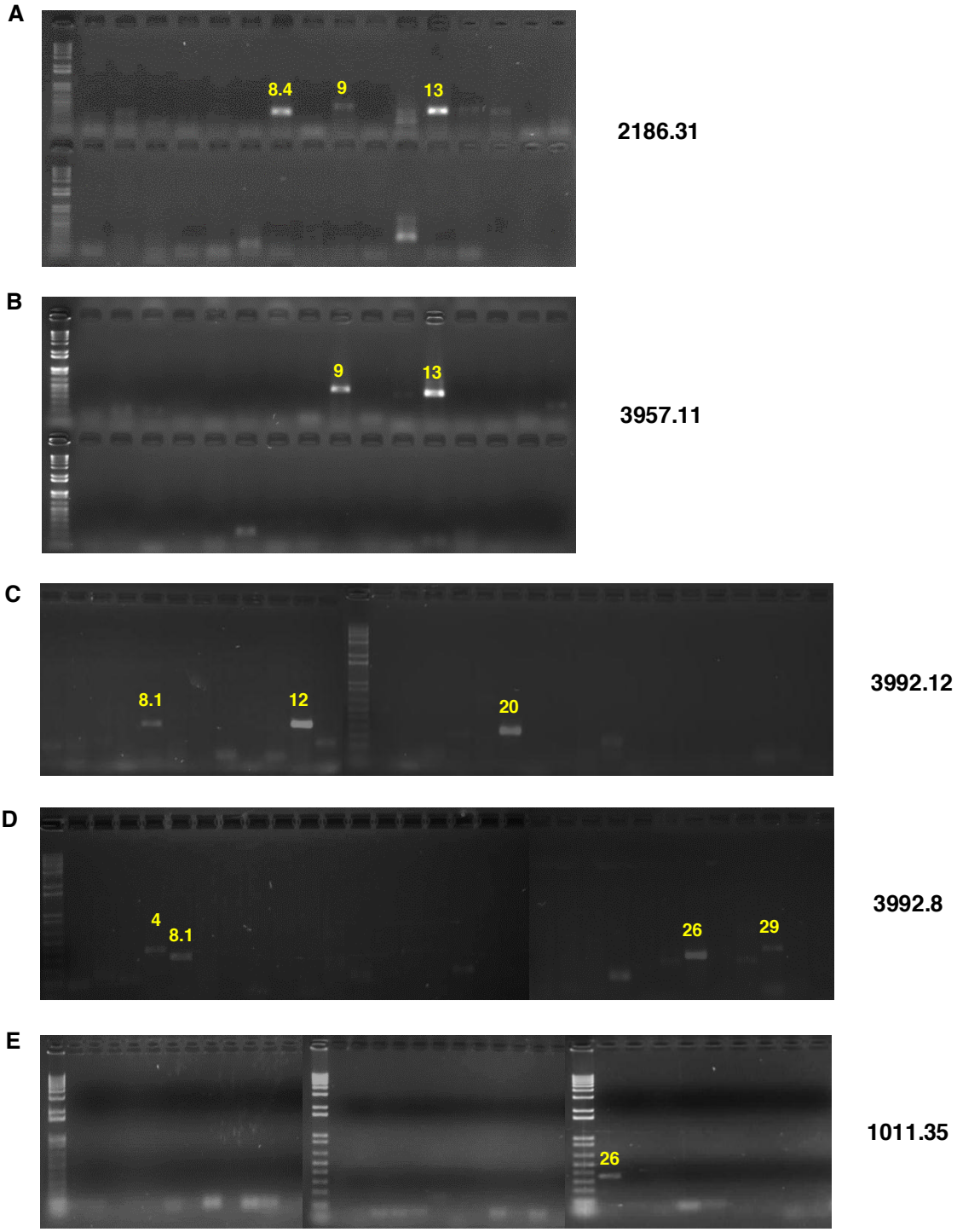


Figure 6.2 Illustration of TCRα expression analysis for $Tp2_{50-59}$ epitope-specific CD8 T cell clones with the defined TCRβ rearrangement.

Single-band PCR products for multiple Vα-specific primers were seen from some CD8 T cell clones including 2186.31 (A), 3957.11 (B), 3992.12 (C) and 3992.8 (D). From CD8 T cell clone 1011.35 (D), only one PCR product was observed and amplified using Vα26-specific primer.

Table 6-3 Sequences of identified TCRα rearrangements for $Tp2_{50-59}$ epitope-specific CD8 T cell clones from different A10-homozygous individuals

Clone	Vα	FR	CDR3	FR	Jα
2186.31	8-f	TGTGCTCTC C A L	CTCCGTTCAGGCTGGCAACTG L R S G W Q L	ACCTTTGGA T F G	22
	13-b	TGTGCAGCA C A A	AGTGACCGATCTAACTACAAGCTC S D R S N Y K L	ACCTTTGGA T F G	20
3992.8	26-ae	TGCATCCTG C I L	ATGGAGAGTTCAGGCTGGCAACTG M E S S G W Q L	ACCTTTGGA T F G	22
3992.12	12-f	TGTGCAGTT C A V	GACAGGGACAAACTC D R D K L	ATCTTTGGG I F G	34
3957.11	13-b	TGTGCAGCA C A A	AGTGCCAGCTACGGCCAGAAC S A S Y G Q N	TATTTTGGG Y F G	49
1011.35	26-i	TGCATCCTG C I L	ATGGCGGGTTATCAGAAACTC M A G Y Q K L	ACATTTGGA T F G	8.2

Both nucleotide and amino acid sequences for CDR3α, Vα and Jα gene usages are shown for defined $Tp2_{50-59}$ epitope-specific CD8 T cell clones from four A10-homozygous individuals. Clone identity is expressed as animal label followed by the clone label generated for the respective animal. Germline Jα gene segments within CDR3α region are underlined.

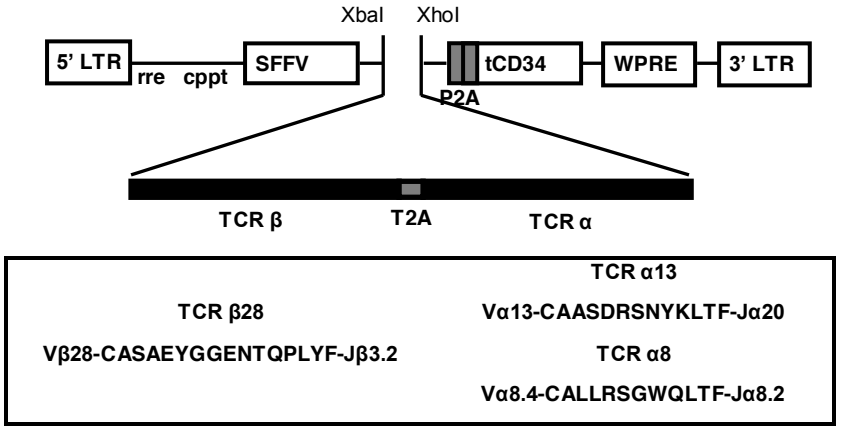
6.3.2 Verification of the $Tp2_{50-59}$ epitope specificity for identified αβTCR heterodimers

Having identified TCRα chains that are co-expressed with the Vβ28-CASAEYGGENTQPLYF-Jβ3.2 TCRβ chain in the $Tp2_{50-59}$ epitope-specific CD8 T cell clones, the epitope specificity of the different TCR α and β chain pairs for the $Tp2_{50-59}$ epitope was investigated. Two constructs were initially generated using the same TCR β chain (TCRβ28) with the two TCRα chains, TCRα8 and TCRα13, identified from the same CD8 T cell clone 2186.31. The initial aim was to determine which of the two α chains conveyed epitope specificity.

6.3.2.1 Generation of lentiviral-bovine TCR vectors

A vesicular stomatitis virus glycoprotein (VSV-g)-pseudotyped HIV-based second-generation lentivirus expression system was employed for *in vitro* expression of the bovine TCR heterodimers. Before constructing lentiviral-TCR vectors, full-length cDNAs of both defined TCR α and β chains were obtained through PCR amplification, cloning and sequencing as described in section 2.3.4.2. The cDNA and amino acid sequences of the selected cDNAs are shown in Appendix C. Figure 6.3 shows a map of the lentiviral-TCR constructs. To obtain equivalent levels of TCR α-

chain and β-chain expression, an oligonucleotide encoding a self-cleaving peptide (T2A) was introduced to link the two full-length TCR cDNA transcripts; this also enabled gene expression to be controlled by the same promoter [Yang et al., 2008, de Felipe et al., 1999]. The lentiviral vector also incorporates a truncated human CD34 gene (tCD34) downstream to the cloning site and linked with the TCR transgene cassette using another oligonucleotide encoding the P2A self-cleaving peptide derived from Porcine Teschovirus-1. The tCD34 gene was introduced as a reporter gene for detecting target gene expression and it also acts as a control for the activity of the target gene promoter. The tCD34 gene has been found as a naturally occurring splice variant of human CD34, with part of the cytoplasmic domain for signal transduction being deleted [Fackler et al., 1995]. Having no interference with physiological functions of target cells, the gene has been used as a maker for selection of engineered primary human and murine T cells [Fehse et al., 2000].



T2A TCCGGCTCCGGAGAGGGCAGGGGGAGCCTGCTCACATGCGGCGATGTGGAGGAGAACCCCGGCCCT
S G S G E G R G S L L T C G D V E E N P G P

P2A TCCGGCTCCGGAGCTACTAACTTCAGCCTGCTGAAGCAGGCTGGAGACGTGGAGGAGAACCCTGGACCT
S G S G A T N F S L L K Q A G D V E E N P G P

Figure 6.3 Lentiviral constructs of bovine TCR. An HIV-based second-generation lentivirus vector was used to express bovine TCR α and β genes. The vector uses a SFFV promoter for driving gene expression and a tCD34 tag for transgene detection. Full-length transcripts of selected TCR α and β chains were obtained through PCR analysis as described in section 2.3.4.2. A single sequence includes a TCR α and a TCR β chain combined by a self-cleaving T2A peptide was designed and cloned into the lentiviral vector. Two lentiviral constructs were generated with two TCR α chains from the same CD8 T cell clone 2186.31.

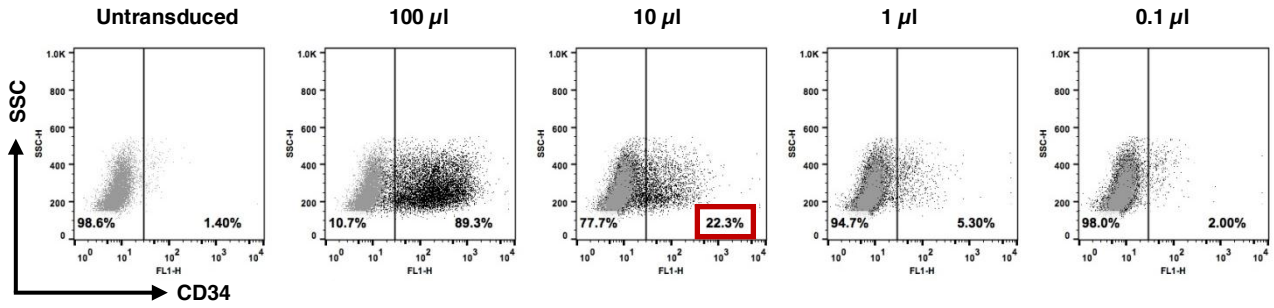
6.3.2.2 Epitope specificity of bovine TCR transgenes

Virus titre and transgene expression of bovine TCR were determined using the TCR^- human leukemia Jurkat T cell line JRT3-T3.5 [Weiss and Stobo, 1984]. As shown in Figure 6.4, CD34 expression was detected for the lentiviral-TCR transgenes incorporating either of the TCR α chains, suggesting successful expression of the bovine TCR gene inserts. Virus titre was determined for both constructs as described in section 6.2.3. As shown in Figure 6.4C, virus titre for the TCR α13β28 construct was one log higher than that for the TCR α8β28 construct.

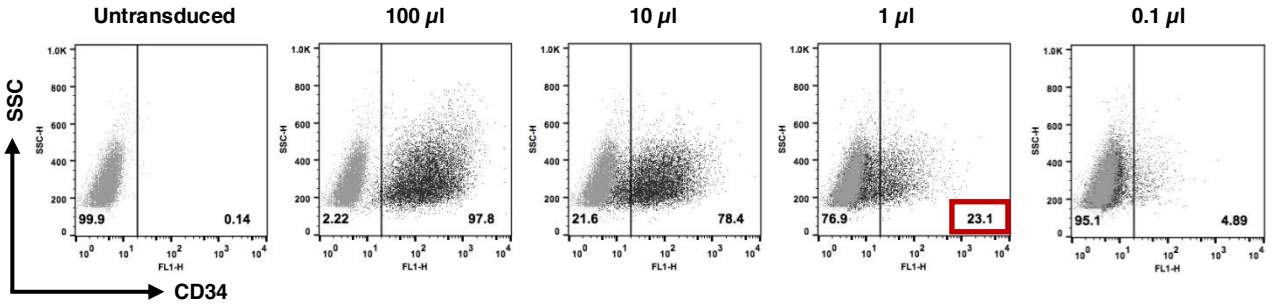
Detection of cell surface expression of the epitope specific TCR transgene was initially examined by staining of the transduced Jurkat cells with 2*01201-$Tp2_{50-59}$ tetramer. The cells were also stained with 2*01201-$Tp2_{49-59}$ and 2*01201-$Tp2_{98-106}$ tetramers as controls. As shown in Figure 6.5, there was no detectable staining with any the tetramers of cells expressing either of the TCR transgenes. Despite the absence of tetramer staining, evidence of functional activity of the expressed TCR transgenes was investigated. Luciferase production was measured for the transduced Jurkat cells following stimulation with antigen presenting cells (APC, 592TA) loaded with peptide for the $Tp2_{50-59}$ epitope. As shown in Figure 6.6, cells expressing either TCR transgenes did not show significant increased luminescence activity upon stimulation with the $Tp2_{50-59}$ epitope, compared with APC alone or APC pulsed with the control $Tp2_{98-106}$ epitope. Stimulation with *T. parva*-infected A10-homozygous cells (592TpM) also did not result in an increase in luciferase activity.

In summary, although the CD8 T cell clones, from which the two TCRα chain were obtained, showed $Tp2_{50-59}$ epitope specificity and gave strong staining with the 2*01201-$Tp2_{50-59}$ tetramer (data not shown), expression in Jurkat cells of transgenes incorporating the two TCR α and β chain combinations detected in the T cell clones did not result in detectable expression of a functional TCR, based either on tetramer staining or responses to antigenic stimulation. The reasons for this result are not clear.

A – TCR α8β28



B – TCR α13β28



C – Virus titer

Lentiviral construct	**Virus titer (TU/ml)**
TCR α8β28	2×10^5
TCR α13β28	2×10^6

Figure 6.4 Lentiviral transduction of Jurkat cells and virus titering. A and B, transduction of JRT-T3.5 cell line with serial diluted viral particles was performed according to section 6.2.3. Percentages of transgene expression cells were analysed using flow cytometry. Un-transduced cells are represented by grey dots and transduced cells are represented by black dots. C, virus titers for both lentiviral constructs were calculated from a dilution generating less than 30% of transgene-expressing cells (marked with red rectangles in A and B).

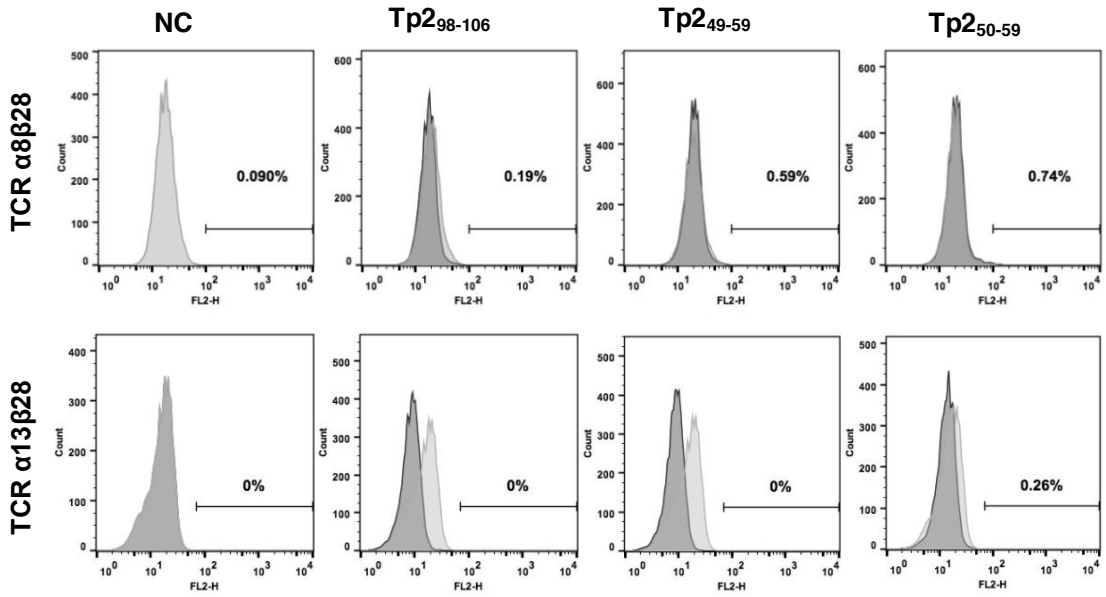


Figure 6.5 Tetramer staining of transduced Jurkat cells to determine epitope specificity of expressed TCRs.

Tetramer staining was performed according to section 2.2.9. Light grey histograms represent un-transduced cells and dark grey histograms represent transduced cells.

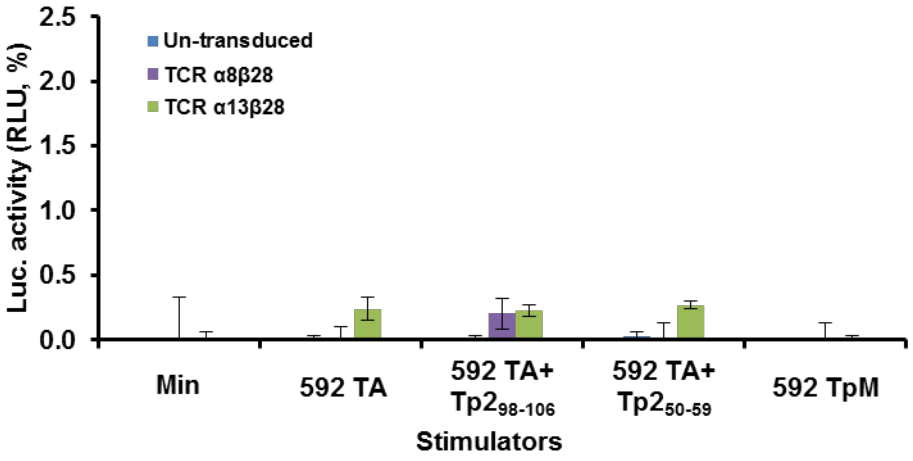


Figure 6.6 Functional activity of TCR transgenes. Luciferase activity of TCR α8β28 and TCR α13β28 transduced JRT3-T3.5 cells were detected to evaluate the functional activity and confirm the epitope specificity of bovine TCR transgenes. Data were obtained from duplicate wells of one test for each condition and presented as average with standard deviation. Both TCR transgenes didn't show luciferase activity to the Tp2$_{50-59}$ epitope.

To investigate whether the failure to detect expression was a consequence of the particular TCRα chains selected, further experiments were undertaken to isolate and express full-length TCRα cDNA from other CD8 T cell clones. CD8 T cell clone 1011.35 was initially chosen as a source of TCRα chain for generating the transgene construct. This T cell clone yielded a PCR product from only one of the TCRα primer pairs tested, suggesting expression of a single α chain. Full-length cDNA of the identified TCRα chain from clone 1011.35 was amplified, cloned and sequenced according to the description in section 2.3.4.2. The obtained TCRα26 (Vα26-CILMAGYQKLTF-Jα8.2) sequence was then constructed into a lentiviral-TCR α26β28 vector as described in the previous section. As shown in Figure 6.7, the TCR α26β28 transgene was successfully expressed following transduction of Jurkat cells. Approximately 85% of the transduced cells expressed CD34, of which about 60% showed specific staining with the 2*01201-$Tp2_{50-59}$ tetramer; no staining was detectable with $Tp2_{98-106}$ and $Tp2_{49-59}$ epitope-specific tetramers. Moreover, $Tp2_{50-59}$ epitope stimulation of TCR α26β28 transgene expressing cells resulted in significant up-regulation of luciferase production comparing to stimulation with the control epitope $Tp2_{98-106}$ ($P<0.05$) or with APC alone ($P<0.01$). These results clearly demonstrated successful expression of bovine TCRα26β28 in Jurkat cells and confirmed it's specificity for the $Tp2_{50-59}$ epitope.

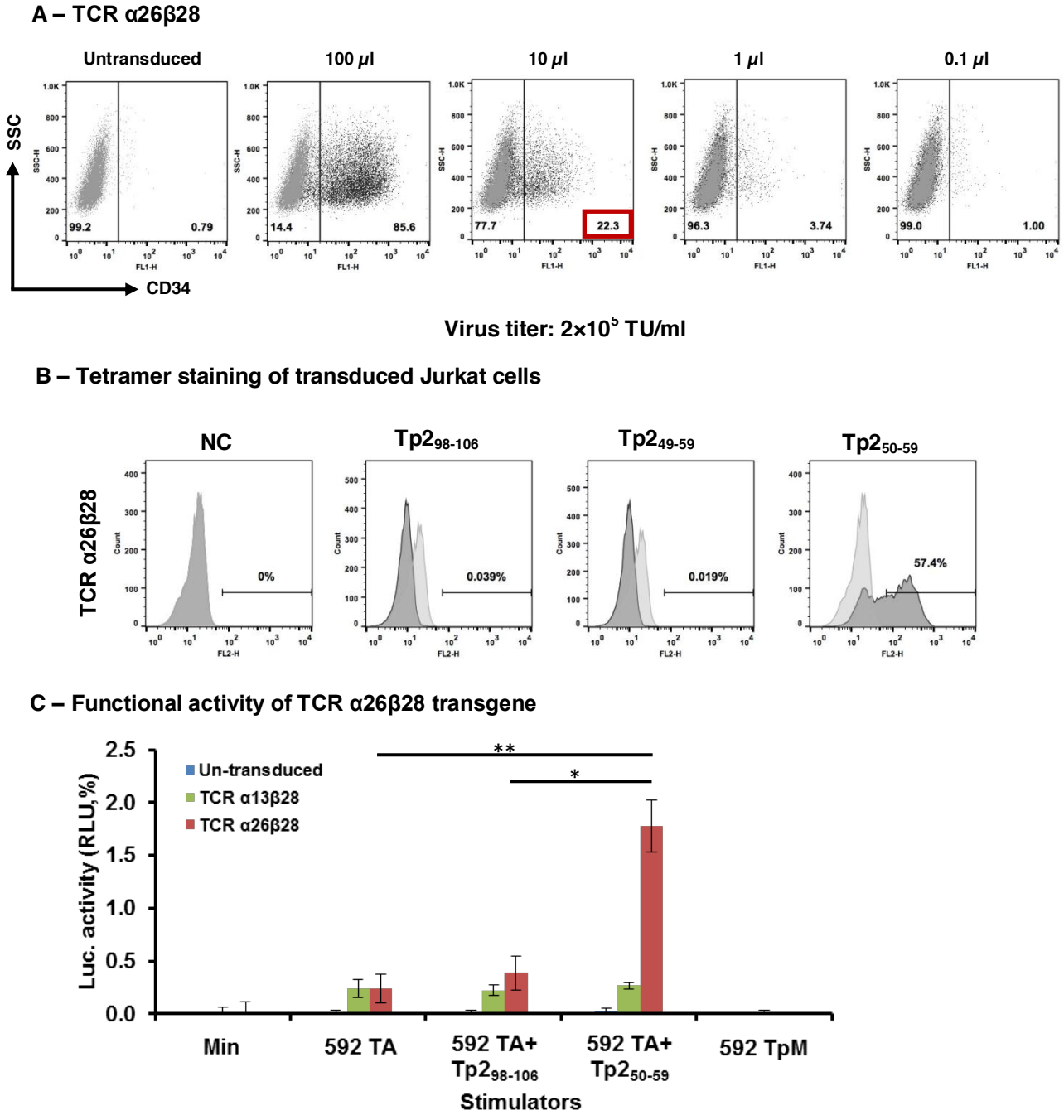


Figure 6.7 The TCRα26β28 transgene expression and its $Tp2_{50-59}$ epitope specificity.

A, the TCRα26β28 transgene expression in transduced (black dots) Jurkat report cell line JRT3-T3.5. Un-transduced cells are shown as grey dots. Virus titer was determined as 2×10^5 TU/ml. B, tetramer staining of transduced Jurkat cells (dark grey histograms) showed the $Tp2_{50-59}$ epitope specificity. Light grey histograms represent un-transduced cells. C, luciferase activity of TCRα26β28 transduced Jurkat cells showed significant increase under the $Tp2_{50-59}$ epitope stimulation, comparing with empty APC ($P<0.01$) and the control $Tp2_{98-106}$ epitope ($P<0.05$). Data were obtained from duplicate wells of one test for each condition and presented as average with standard deviation.

6.3.3 Lentiviral transduction of bovine CD8 T cells for epitope-specific TCR expression

Verification that the bovine TCRα26β28 clonotype can be expressed in Jurkat cells and retains functional specificity for the immunogenic epitope $Tp2_{50-59}$ provided the basis to investigate *in vitro* transduction of bovine naïve CD8 T cells. However, the efficiency of lentiviral transgene system in bovine immune cells is still unknown. It is well established that lentiviral transduction is ineffective in resting cells. Hence, a protocol for pre-activating CD8 T cells was investigated before proceeding with lentivirus transduction.

6.3.3.1 Activation of bovine CD8 T cells for lentiviral transduction

Activation of bovine CD8 T cells purified (by MACS purification) from PBMC of both *T. parva* immunized and naive healthy cattle was carried out using anti-CD3 (1µg/ml) and anti-CD28 (5µg/ml) monoclonal antibodies as described in section 6.2.5. After 20 hours stimulation, cell cycles of un-stimulated and stimulated cells were analysed through DNA staining with Hoechst 33285 dye. According to the DNA content, cell cycle (approximately 24 hours) for a typical eukaryotic cell can be divided into four discrete phases: M (mitosis, 4N), G1 (gap 1, cells in resting or preparing for DNA synthesis, 2N), S (synthesis, DNA replication, 2N-4N), and G2 (gap 2, protein synthesis for mitosis, 4N) [Callard and Hodgkin, 2007]. As shown in Figure 6.8, in the absence of stimulation the cells only showedminimum level of cell activation, with more than 70% cells staying in G0/G1 (2N) phase after 20 hours culture in standard cell culture media. A small percentage (2.5%) of cells entered G2/M phase (4N). By contrast, nearly 60% cells entered G2/M phase after 20 hours stimulation with the anti-CD3 and anti-CD28 and 3.6% cells stayed in G0/G1 phase. More of the stimulated CD8 cells (38%) were in S phase than the un-stimulated cells (26%). These results confirmed the activation of bovine CD8 T cells by stimulation via the TCR (anti-CD3) and the co-stimulatory ligand CD28.

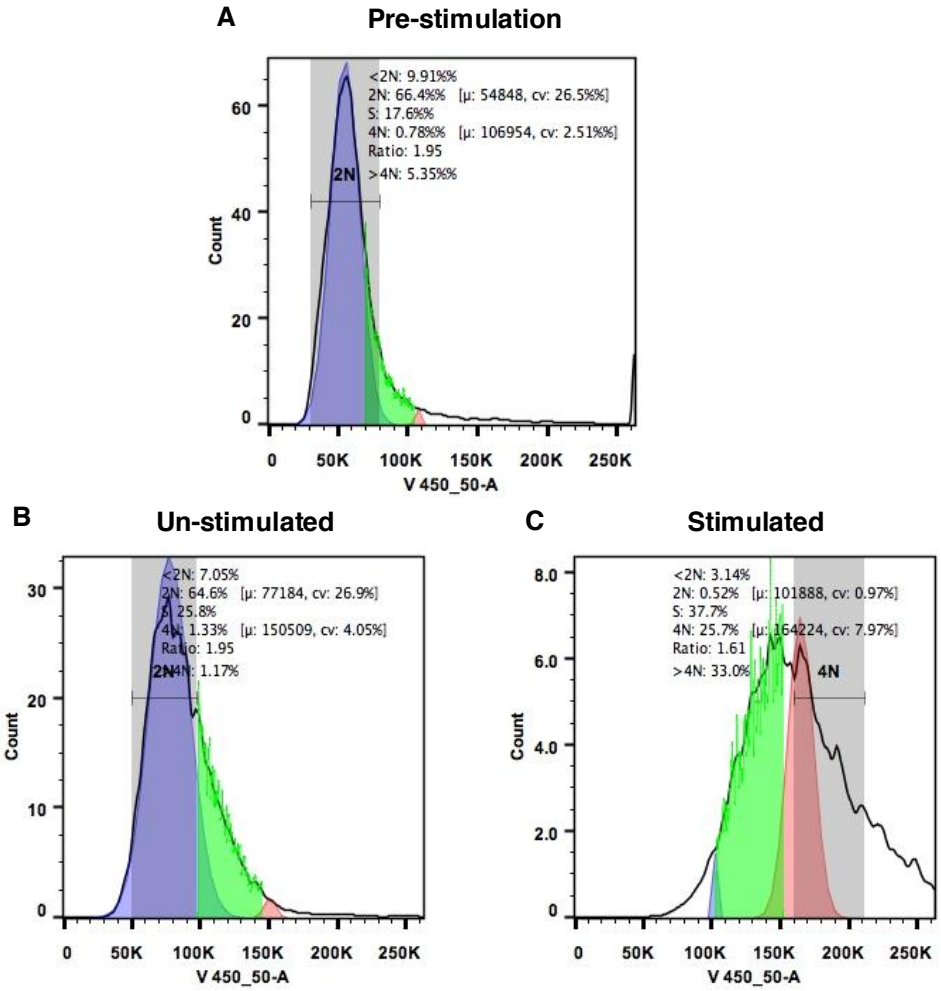


Figure 6.8 Compare cell cycles of un-stimulated and stimulated bovine CD8 T cells. Purified bovine CD8 T cells were cultured with or without TCR stimulation for 20 hours and then stained with Hoechst 33285 for cell cycle analysis. A, cell cycle analysis of CD8 T cells before stimulation, 76% cells in G0/G1 phase (2N, blue), 17.6% cells in S phase (green) and 6% cells in G2/M phase (4N, red). B, for un-stimulated cells, >70% of cells in G0/G1 phase, a small amount increase was seen for S phase cells (25.8%) and only 2.5% cells in G2/M phase. C, for stimulated cells, proportion increased for G2/M phase cells (58.7%) and only 3.6% cells remained in G0/G1 phase, nearly 40% cells in S phase after stimulation.

6.3.3.2 Lentiviral transduction of pre-activated bovine CD8 T cells

A preliminary experiment was carried out to transduce bovine CD8 T cells with lentiviral particles incorporating the TCRα26β28 transgene, after 20-24 hours stimulation (section 2.2.14). Transgene expression was monitored by examining cell surface expression of the reporter gene product tCD34 at 48 and 72 hours after transduction. The level of transgene expression appeared to be higher at 48 hours than at 72 hours. Therefore, transgene expression was detected at 48 hours after transduction of bovine CD8 T cells. The expression of CD34 on the lentiviral transduced bovine CD8 T cells is shown in Figure 6.9. Cells transduced with pseudotyped lentivirus constructed with bovine TCRα13β28 transgene showed CD34 expression on 23% of the cells. However, only 2% of the CD8 T cells transduced with TCRα26β28 transgene were detected as CD34 positive. It has been shown that virus titre for the TCRα26β28 construct was one log lower than that for the TCRα13β28 construct, which determines that MOI for transduction couldn't reach to the same level for the two constructs. As shown in Figure 6.9, MOI for the TCRα13β28 construct was 10 for transduction, while it was 5 for the TCRα26β28 construct. This lower MOI might be the reason for lower level of TCRα26β28 transgene expression.

Staining of the CD8 T cells with the 2*01201-$Tp2_{50-59}$ tetramer was also carried out to detect cells expressing a $Tp2_{50-59}$–specific TCR. As shown in Figure 6.10, tetramer-positive cells were not detected in CD8 T cells transduced with either of the TCR transgenes. The results obtained with the CD8 T cells transduced with TCRα13β28 are consistent with those obtained with transduced Jurkat cells, which showed CD34 expression, but failed to stain with tetramers. For the TCRα26β28 transgene, failure to detect tetramer positive cells may be due to the low expression level, as assessed by CD34 expression.

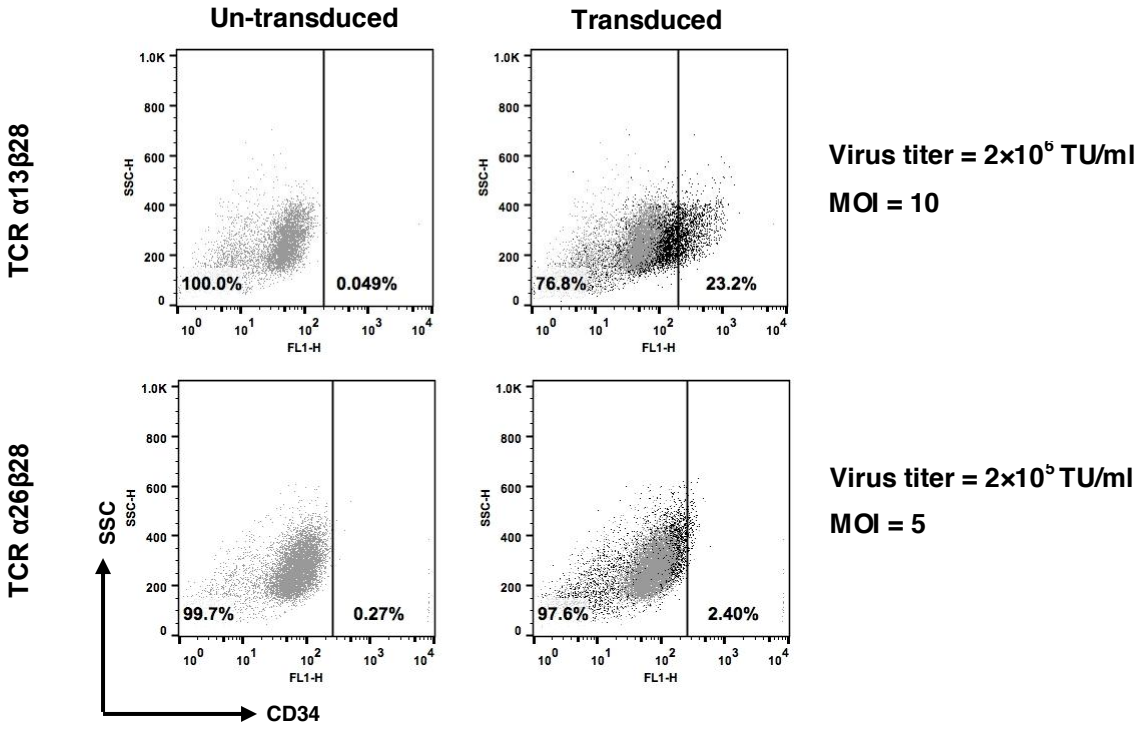


Figure 6.9 TCR transgene expression in lentiviral transduced bovine CD8 T cells.
Activated bovine CD8 T cells were transduced with transgenes TCRα13β28 (up panel) and TCRα26β28 (low panel). Transgene expression was detected using anti-CD34 antibody. Dot plots of FACS analysis are shown. Grey dots represent un-transduced cells and black dots represent transduced cells. Virus titre and multiplicity of infection (MOI) are shown for both lentiviral constructs.

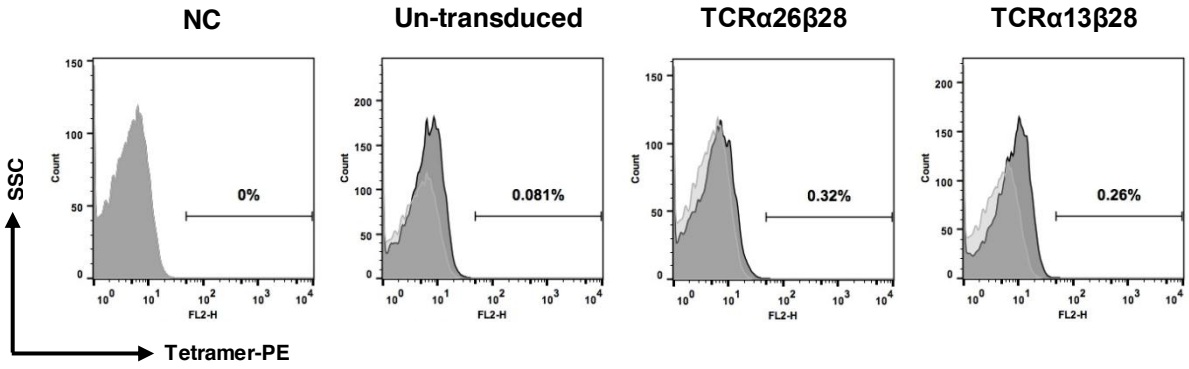


Figure 6.10 The $Tp2_{50-59}$ epitope-specific tetramer staining of transduced bovine CD8 T cells.
Histogram of FACS analysis are shown. Light grey histograms represent unstained cells and dark grey histograms represent stained cells. Tetramer positive populations were not detected from bovine CD8 T cells transduced with any of the two αβTCR transgenes.

6.4 Discussion

Appropriate functional differentiation of naïve antigen-specific CD8 T cells is believed to be critical for bovine CD8 T cell responses against *T. parva* infection. However, limited information is available for factors involved in the induction of *T. parva*-specific CD8 T cell responses. One limitation for further investigation is to get access to naïve antigen-specific CD8 T cells, which are generally present at extremely low frequency in the naïve pool. Therefore, the current study was setup aiming to evaluate the feasibility of generating bovine naïve antigen-specific CD8 T cells *in vitro*.

For this purpose, the experiments described in this chapter have identified several TCRα chains from CD8 T cell clones specific for the $Tp2_{50-59}$ epitope, which express a conserved TCRβ chain. The use of a TCR clonotype that is conserved between animals would potentially allow transfer of *in vitro* expanded antigen-specific transduced CD8 T cells into animals. Three pairs of TCR α and β chains were constructed into lentivirus-TCR constructs for transgene expression on target cells (i.e. bovine naïve CD8 T cells). Using a TCR^- human T lymphocyte reporter cell line, the epitope specificity for one of the αβTCR construct was confirmed by tetramer staining and demonstration of functional activity. The identification of this functional αβTCR provides an important material for transferring antigen-specificity into bovine naïve CD8 T cells. Preliminary experiments, using lentivirus-TCR constructs, showed successful transduction of bovine CD8 T cells for TCR transgene transcription, although the level of transduction obtained with the functional construct was very low to achieve functional activity, probably because of insufficient lentivirus titre. These results indicate that with future optimization, the lentiviral transgene expression system would be suitable for delivering the defined TCR transgenes into bovine naïve CD8 T cells.

For the identification of TCRα chains paired with the defined TCRβ clonotype, a PCR approach using a panel of Vα subgroup specific primers, designed and provided by Dr. Tim Connelley [Connelley et al., 2014], was applied to a number of CD8 T cell clones derived from 4 A10-homozygous cattle. Previous studies have

demonstrated that bovine TCRα repertoire contains many subgroups with multiple members of genes that have arisen by gene duplication [Connelley et al., 2014, Reinink and Van Rhijn, 2009]. The design of primers that will reliably amplify all members of a duplicated subgroup and retain specificity for that subgroup is difficult. To achieve the former, it was necessary in some cases to accept cross-reactivity of primer sets with members of other groups. Therefore, the identity of the amplified TCRα chain V subgroups could be deduced with certainty from sequencing of the PCR products. This lack of subgroup specificity accounted for the detection of clones that yielded PCR products with more than two primer sets. Sequencing of PCR products obtained from individual T cell clones revealed cross-reactivity of the Vα9 PCR primer with a member of Vα13 subgroup, the Vα26 PCR primer with Vα4. As observed in other species [Han et al., 2014, Padovan et al., 1993], examples of clones exhibited two TCRα chain rearrangements, both of which were full open reading frames (e.g. 2186.31), were found. Single TCRα rearrangements were identified for other examined clones and different individuals tend to have different TCRα chain usages. The observation of two CD8 T cell clones from the same animal (403992 and both can pair with the same TCRβ chain) having different TCR α chains indicates that the component of the $Tp2_{50\text{-}59}$-specific CD8 T cells expressing the Vβ28-CASAEYGGENTQPLYF-Jβ3.2 β chain rearrangement in this animal arose from at least two independently generated clones in the naïve T cell repertoire.

Expression of the same two recombined TCRα chains was observed for multiple clones from animal 302186. This would imply that these clones are highly likely to have arisen from the same rearranged CD8 T cell in the naïve T cell repertoire. Since only one TCRα rearrangement is likely to form the functional αβTCR heterodimer specific for the $Tp2_{50\text{-}59}$ epitope, experiments were undertaken to identify which TCRα chain conveyed the functional specificity and thus could be used for transgene expression. A third TCRα chain, which is expressed by CD8 T cell clones from animal 1011 and only yielded a PCR product with one of the Vα subgroup PCR primers, was chosen for the transduction experiments. For generating lentiviral-TCR constructs, full-length cDNA for TCR α and β chains were obtained and sequenced to confirm that no PCR errors were introduced into the constructs.

A TCR$^-$ transformed human T cell line, referred to as Jurkat cell line JRT3-T3.5, was initially used for transgene expression. The cell line expresses a luciferase reporter gene, under the transcriptional control of NFAT (nuclear factor of activated T cells)-responsive elements, which is activated upon stimulation via the TCR resulting in expression of the luciferase gene [Aarnoudse et al., 2002]. Both tetramer staining and luminescence activity of transduced reporter cells were used to determine the epitope specificity and functionality of the three αβTCR transgenes. The first two pairs of αβTCR heterodimer didn't elicit detectable responses to the defined $Tp2_{50-59}$ epitope, while the TCRα26β28 construct (from CD8 T cell clone 1011.35) showed clear specificity for the $Tp2_{50-59}$ epitope from both tetramer staining and the functional assay. Nearly 60% of the TCRα26β28 transduced Jurkat cells were positively stained with the $Tp2_{50-59}$ tetramer (Figure 6.7B). Moreover, transduced cells showed specific activation in response to the $Tp2_{50-59}$ epitope, but not to the control epitope or APC alone (Figure 6.7C). Confirmation of epitope specificity and functional competence of the TCRα26β28 construct provided proof of concept for further experiments to transduce bovine CD8 T cells.

The reasons are unclear for lack of functional expression of the other two α/β TCR constructs, which utilised two apparently functional α chains from the same CD8 T cell clone. However, lack of CD8 expression on the reporter cells and/or possibly low TCR affinity might result in the failure of detection, since both tetramer staining and activation of some T cells is dependent on CD8 [Laugel et al., 2007, Wooldridge et al., 2005]. In a study using tetramers incorporating modified MHC I heavy chains unable to interact with CD8, epitope-specific CD8 T cells with high functional avidity TCRs could be detected but not CD8 T cells with low avidity TCRs [Choi et al., 2003].

Nevertheless, identification of one bovine αβTCR heterodimer with antigen specificity and functional responsiveness allowed initiation of preliminary experiments to transduce bovine CD8 T cells. The efficiency of TCR transgene expression using lentiviral transfer system was primarily examined in activated CD8 T cells. Successful transduction of bovine CD8 T cells at the transcriptional level, based on expression of the CD34 reporter gene, was confirmed for the construct

TCRα13β28. However, expression of a functional TCR on the cell surface of the transduced CD8 T cells was not detectable by tetramer staining. This is consistent with the tetramer staining result of TCRα13β28 transduced Jurkat cells. For the TCRα26β28 construct with confirmed functional specificity to the $Tp2_{50-59}$ epitope, low level (2%) of transgene expression was detected on transduced CD8 T cells. Potential reason could be the lower virus titre (a log lower than the virus titre of TCRα13β28 construct), which resulted in lower MOI for CD8 T cell transduction. Functional TCR was also not detected from TCRα2628 transduced CD8 T cells by tetramer staining. Except for the low level of transgene expression, other factors including mis-pairing with endogenous TCR chains and competition with endogenous TCRs for surface expression could all interfere with the functional detection of expressed TCR transgene [Daniel-Meshulam et al., 2012, Okamoto et al., 2009]..

For future studies, optimisation of virus production conditions to increase virus titre would be necessary. It has been reported that various factors, including time of harvest after transfection, lentiviral vector stability, cell type and transfection methods and use of medium additives, can influence the yield of lentivirus obtained [Ansorge et al., 2010]. In terms of the efficiency of lentiviral transduction of CD8 T cells, various strategies have been proposed to improve TCR transgene stability and correct pairing, such as introducing a second disulphide bond into the constant regions of TCR α and β chains to enhance pairing, knocking down expression of the endogenous TCR chains to avoid pairing with the expressed transgenes and increasing CD3 expression [Daniel-Meshulam et al., 2012]. In a recently developed system, human hematopoietic progenitor cells ($CD34^+$ cells) were used for transduction of antigen-specific TCRs and agonist peptides were used to inhibit endogenous TCR expression of transduced cells. This method showed high efficiency with nearly all generated naïve CD8 T cells expressing the single TCR transgene [Snauwaert et al., 2014]. Previous work from Niku *et al* has produced an anti-bovine CD34 antibody, which was later used to identify a CD34 expressing population with features of hematopoietic progenitor cells from bovine foetal and adult bone marrow [Niku et al., 2007, Pessa-Morikawa et al., 2012]. Building on

these developments, future work exploring the usage of bovine T cell progenitors for TCR transgene expression would be of help to generate bovine naïve antigen-specific CD8 T cells *in vitro*.

Overall, results in this chapter present the identification of a first reported bovine αβTCR heterodimer with functional specificity to the Tp2 antigen of *T. parva*. Although extensive work will be required for final generation of bovine naïve antigen-specific CD8 T cells, studies in this chapter provide the necessary material and evidence for applying lentiviral transduction approach to bovine T cells.

Chapter 7: General discussion

Previous studies demonstrated that the *T. parva* antigen Tp2 induces highly dominant CD8 T cell responses in BoLA-A10 homozygous cattle immunised by infection and treatment against *T. parva* [MacHugh et al., 2009]. Three epitopes presented by the same MHC I allele, BoLA 2*01201, have been identified in this antigen. This project was set up to: (i) investigate the immunodominance hierarchy of these three Tp2 epitopes in A10+ cattle and to determine the predictability of the antigenic specificity of CD8 T cell responses in a larger number of animals; (ii) examine the clonal composition of the epitope-specific populations by employing TRB gene sequencing, to determine the diversity of the responses and whether the TRB chain rearrangements show any evidence of conservation; (iii) establish protocols for expressing functional TCRs in cultured cells by lentivirus transduction with cloned full-length TCR α and β chain cDNAs. Results obtained from these studies had the following significant outcomes.

(1) A marked difference in the epitope dominance hierarchy detected in A10-homozygous and A10-heterozygous cattle. The Tp2 epitope specificity of CD8 T cell responses were examined in 11 cattle, including 5 A10-homozygous and 6 A10-heterozygous animals, by generation of CD8 T cell lines and staining with class I MHC tetramers. The responses of the A10-homozygous animals were remarkably consistent, all 5 animals responding to all 3 epitopes with the $Tp2_{49\text{-}59}$ epitope consistently the most dominant. By contrast, the 6 A10-heterozygous animals failed to respond to the $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitopes and 2 of the animals did not have detectable responses to any of the 3 defined Tp2 epitopes. Analyses of the *in vivo* responses in 2 homozygous and 2 heterozygous animals showed that epitope dominance hierarchy reflected that observed *in vitro* and demonstrated that CD8 T cells specific for the dominant epitopes represented 2-8% of the CD8 T cells at the peak of the response. Attempts to detect epitope-specific T cells in the two animals that did not have a detectable response to Tp2, by sorting of low frequency CD8 T cells with pMHC I tetramers, did not detect any positive cells. Based on these experiments, it was estimated that epitope-specific CD8 T cells (if present) are at a frequency of less than $1/10^5$. These results clearly demonstrate that the co-expression

of other MHC I alleles along with those on the A10 haplotype profoundly alters the specificity of the response, such that the A10-heterozygous animals examined were unable to generate CD8 T cell responses against the $Tp2_{49-59}$ and $Tp2_{50-59}$ epitopes.

(2) TRB repertoire analyses detected markedly expanded clonotypes within the specific responses and identified TRB gene rearrangements that were conserved or partially conserved between animals. Analyses of the sequences of TRB chains expressed by the epitope-specific CD8 T cells, following amplification by a template-switch anchored RT-PCR, demonstrated a broadly similar population structure for the different specificities, each population containing 1-3 highly expanded dominant TRB clonotypes along with variable numbers of low-abundant clonotypes. Of particular interest was the detection of semi-conserved TRB clonotypes in the $Tp2_{98-106}$-specific CD8 T cell populations of different animals and a completely conserved TRB clonotype, expressing the Vβ28-CASAEYGGENTQPLYF-Jβ3.2 rearrangement, in the $Tp2_{50-59}$-specific populations from all 5 A10-homozygous cattle examined.

(3) Establishment of a method to detect epitope-specific CD8 T cells *ex vivo* by high-throughput sequencing of TRB. The use of TRB clonotype analyses to detect epitope-specific T cells in different immune individuals is hampered due to the diversity and generally 'private' TRB specificities within CD8 T cell repertoires. Using the identified 'public' TRB sequence as a marker for $Tp2_{50-59}$-specific CD8 T cell populations, an attempt was made to use HTS analysis to examine the potential role of TCR repertoires in alterations of immunodominance hierarchies – a potential explanation for the failure of A10-heterozygous cattle to respond to this epitope $Tp2_{50-59}$. Although technically successful the results from a validation trial suggested that the methodology would not enable definitive conclusions to be made.

(4) Reagents and methods established to generate cultured cells expressing functional epitope-specific TCRs. Several functional TCR α chains co-expressed with the conserved Vβ28-CASAEYGGENTQPLYF-Jβ3.2 β chain in CD8 T cell clones specific for the $Tp2_{50-59}$ epitope were identified and lentivirus constructs were generated with three of the α and β chain pairs to investigate expression. Lentivirus

transduction of Jurkat cells resulted in expression of all 3 gene pairs and one construct (TCRα26β28) was confirmed to express functional TCR on the cell surface, confirmed by responsiveness to the $Tp2_{50-59}$ epitope. This is the first report of successful expression of a functional bovine αβTCR by lentivirus transduction. Preliminary experiments of lentiviral transduction of bovine CD8 T cells demonstrated transgene expression by the transduced cells, confirming successful introduction of the genes into bovine CD8 T cells, but there was insufficient time to fully investigate surface expression of the functional proteins.

Results of this project provided clear quantitative data on the epitope dominance hierarchy of the three defined Tp2 epitopes in *T. parva*–immunised Holstein cattle expressing the A10 MHC I haplotype. The findings represent progress towards understanding the immunodominant CD8 T cell responses against *T. parva*. Further investigation will be required to discover the mechanistic basis of the Tp2 epitope dominance hierarchy.

One question raised from the observed difference in epitope dominance between A10-homozygous and -heterozygous cattle is whether it reflects a difference in antigen processing that alters presentation of the defined Tp2 epitopes in parasite infected A10-heterozygous cells. Proteasome activities, TAP and endoplasmic reticulum aminopeptidase (ERAP) have all been demonstrated to influence the generation of epitopes [Tenzer et al., 2009]. Future experiments to examine the ability of parasitized A10-heterozygous cells to generate and present $Tp2_{49-59}$ and $Tp2_{50-59}$ epitopes, and to determine the epitope abundance on cell surface, will be required to understand the effect of non-restriction MHC I haplotypes on epitope processing.

The frequency of antigen-specific T cell precursors in the naïve TCR repertoire, the clonotype composition of the repertoire and the differentiation of responding CD8 T cells to establish memory populations, have all been reported to have profound influences on CD8 T cell responses against a number of viruses [La Gruta and Turner, 2014, Appay et al., 2008]. Analyses of the TCRβ chains expressed by samples of epitope-specific CD8 T cells in the current study revealed biased Vβ13.5

gene usage by T cells specific for the $Tp2_{98-106}$ epitope and a conserved TRB clonotype specific for the $Tp2_{49-59}$ epitope. However, to more fully understand the role of TCR repertoire in determining epitope dominance hierarchy and the difference between A10-homozygous and –heterozygous cattle, further experiments are required to address the following questions.

(i) What are the frequencies of CD8 T cell precursors specific for the three defined Tp2 epitopes in the naïve pool of CD8 T cells in A10-homozygous and –heterozygous animals and do the frequencies correlate with the epitope specificities of the responses induced by immunisation? There is evidence that the frequency of naïve T cell precursors can be a predictor of the magnitude of epitope-specific CD8 T cell responses in several virus disease models [Tan et al., 2011, Kotturi et al., 2008]. Techniques such as direct pMHC I tetramer enrichment [Obar et al., 2008] or indirect approach by comparing the expansion rate of endogenous and adoptively transferred titrated naïve epitope-specific CD8 T cells [Blattman et al., 2002] would allow the quantification of naïve CD8 T cell precursor for a given *T. parva* epitope.

(ii) Does the diversity of TCR clonotypes within the repertoires of responding CD8 T cells influence the immunodominance hierarchy of the three defined Tp2 epitopes? A direct correlation between TCR diversity and immunodominance status of viral derived epitopes has been reported in responses to human and mouse pathogens [Balamurugan et al., 2010, Messaoudi et al., 2002]. Current approaches involving fluorescence-activated cell sorting of cells stained with peptide-MHC I multimers (tetramer or dextramer) allow rapid purification of epitope-specific CD8 T cells present at low frequencies [Tario et al., 2015, Wooldridge et al., 2009]. The development of PCR and sequencing techniques, such as the template-switch anchored RT-PCR, single-cell PCR and next-generation sequencing, now facilitate greater depth analysis of TCR repertoires [Shugay et al., 2014, Han et al., 2014]. With the establishment of these methods, a complete view of antigen-specific CD8 T cell repertoires can be achieved.

(iii) What factors are involved in the activation and differentiation of antigen-specific CD8 T cell precursors and how do they influence the epitope

dominance hierarchy? The importance of cross-priming, predominantly mediated by dendritic cells (DCs), for induction of primary CD8 T cell responses against viral infections is well recognised [Smed-Sorensen et al., 2012, Wilson et al., 2006, Heath and Carbone, 2001a]. A recent study of human CD8 T cell responses against HIV antigens demonstrated that cross-presentation of viral antigens by DCs and macrophages favoured CD8 T cell responses to immunodominant epitopes [Dinter et al., 2015]. Work is being carried out by other members of the Roslin group to establish an *in vitro* model to investigate the functional role of DC-mediated cross-presentation to *T. parva*-specific CD8 T cells. Access to bovine naïve CD8 T cells would allow the requirements for the activation of *T. parva* antigen-specific CD8 T cell responses to be investigated using this cross-priming model.

Overall, work in the current study showed clear evidences for epitope dominance variation among cattle expressing the restriction MHC I haplotype. The unique feature of this system, i.e. three epitopes derived from the same antigen and presented by the same MHC I allele, indicates the major influence of antigen-specific CD8 T cells on epitope dominance variation. The verification of a conserved TCRβ chain specific for the $Tp2_{50\text{-}59}$ epitope enables the detection of epitope-specific CD8 T cells directly *ex vivo* from purified CD8 T cells, although the method still requires further validation. With the identified bovine αβTCR heterodimer specific for the Tp2 antigen of *T. parva*, future optimization of the lentivirus production and attempt to transduce bovine naïve CD8 T cells or haematopoietic progenitor cells would greatly facilitate the *in vitro* generation of bovine naïve antigen-specific CD8 T cells.

Appendices

Appendix A. Solutions and medium

Standard Culture Medium (SCM)

RPMI 1640 medium + 25mM HEPES + L-glutamine (Life Technologies, UK)

10% (heat inactivated) foetal bovine serum (Life Technologies, UK)

100U/ml penicillin, 100µg/ml streptomycin, 293µg/ml L-glutamine (penicillin-streptomycin-glutamine 100 x solution) (Life Technologies, UK)

5 x 10^4M 2-mercaptoethanol

RBC Lysis Buffer

Trizma base	10.6g	(0.175M)

0.175M TRIS pH adjusted to 7.4 with concentrated HCl and made up to a volume of 0.5L with Double-distilled water (DDW)

Ammomium chloride	8.55g	(0.16M)

Made up to a volume of 1L with DDW.

RBC lysis buffer consists of 1 part 0.175M TRIS pH7.4 and 9 parts 0.16M ammonium chloride. Filter sterilized using 0.45µmminisart single-use filter (Sartorius, Geottingen, Germany)

FACS Medium

RPMI 1640 medium + 25mM HEPES + L-glutamine (Life Technologies, UK)

2% (heat inactivated) foetal bovine serum (Life Technologies, UK)

0.2% Sodium Azid

Phosphate Buffered Saline (PBS)

Sodium chloride	170.0mM
Potassium chloride	3.4mM
Sodium phosphate, dibasic	9.2mM
Potassium phosphate, monobasic	1.8mM

pH6.8

MACS Medium

PBS + 0.5% bovine serum albumin (BSA) + 2mM EDTA

DMEM Medium

DMEM medium + 4.5g/L D-Glucose + L-glutamine (Life Technologies, UK)

10% (heat inactivated) foetal bovine serum (Life Technologies, UK)

100U/ml penicillin, 100µg/ml streptomycin, 293µg/ml L-glutamine (penicillin-streptomycin-glutamine 100 x solution) (Life Technologies, UK)

SOC Broth (Super Optimal broth with Catabolite repression)

Tryptone	20.00g
Yeast extract	5.00g
Sodium chloride	0.50g
Potassium chloride	0.19g
Glucose	4.00g

pH adjusted to 7.0 with 5M sodium hydroxide, made up to a volume of 1L with DDW and autoclaved

Loading Buffer for Agarose Gel Electrophoresis

15% w/v Ficoll (Type 400) in DDW with bromophenol blue (0.25 v/v) and xylene cyanol (0.25%) dyes

Tris-acetate/EDTA electrophoresis buffer (TAE) 50× stock

Trizma base	242g	(2M)
Glacial acetic acid	57.1ml	(2M)
EDTA	100ml of 0.5M solution	(50mM)

LB Broth

Tryptone	10.00g
Yeast extract	5.00g

Sodium chloride 10.00g

pH adjusted to 7.0 with 5M sodium hydroxide, made up to a volume of 1L with DDW and autoclaved

LB Agar plates

LB broth added with Agar (15g/L) pH adjusted to 7.0 with 5M sodium hydroxide made up to a volume of 1L with DDW and autoclaved

Upon use, LB agar was melted and ampicillin added to give a final concentration of 100µg/ml. approximately 20mls of media was poured into sterile Petri dishes and allowed to set and dry. If LB/IPTG/X-Gal plates were required for blue/white colony selection, 20µl of 0.1M IPTG (Isopropyl β-D-1-thiogalactopyranoside) and 20µl of 50mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) were applied to the surface of the plate and allowed to dry before application of any culture.

Appendix B. Sequences of TRB clonotypes identified from Tp2 epitope-specific CD8 T cell populations.

Sequences of TRB repertoires specific for the 3 defined Tp2 epitopes derived from three A10-homozygous A) and three A10-heterozygous B) cattle are shown in the standardised format: identified Vβ and Jβ gene segments, flanking sequences of CDR3β and CDR3β itself. Number and frequency of encoding nucleotide sequences for each clonotype are shown. Dominant clonotypes for each of the nine CD8 T cell populations are shaded. Nucleotides and amino acid substitutions within germline gene component of CDR3β region are in red font and substitutions within junction regions of CDR3β are in purple font and underlined. Germline Jβ gene components of CDR3β are underlined. Germline Dβ gene components of CDR3β are in blue font. Sequences with the same Vβ and Jβ gene segments but different CDR3β sequences are in brown font. The one clonotype identified from both $Tp2_{49\text{-}59}$ and $Tp2_{50\text{-}59}$ epitope-specific CD8 T cell populations from animal 403957 is in green font.

A) Sequences of Tp2 epitope-specific TRB clonotypes from A10-homozygous cattle

Epitope	Sequence					Number of sequence	Frequency (%)	Animal
	Vβ	FR	CDR3β	FR	Jβ			
$Tp2_{49-59}$	14	TGTGCCAGC C A S	AGTCGGTACGCAGGGCCAGAGACGCTG S R Y A G P E T L	TACTTCGGC Y F G	2.4	25	34.2	302186
	16	TGTGCCAGC C A S	AGCCTACAGCCTTTCTATGACTAT S L Q P F Y D Y	CACTTCGGC H F G	1.2	43	58.9	302186
	16		AGCCTACAGCCTTTCTATGTCTAT S L Q P F Y V Y		1.2	4	5.5	302186
	16		AGCCTACAGCCTTTCTATGGCTAT S L Q P F Y G Y		1.2	1	1.4	302186
	4.3	TGCAGTGCT C S A	ACGAGAGGAGGACGGGAGGACTAT T R G G R E D Y	CACTTCGGCH F G	1.2	2	2.4	403992
	14	TGTGCCAGC C A S	AGTGAATGGAACACAAATGGGCCCCTG S E W N T N G P L	TACTTTGGA Y F G	3.2	33	39.8	403992
	14		AGTGGATGGAACACAAATGGGCCCCTG S G W N T N G P L		3.2	1	1.2	403992
	28	TGTGCCACG C A T	GCTGCAGATCTCGACGACAACCCTCTG A A D L D D N P L	TATTTTGGA Y F G	3.3	46	55.4	403992
	28	TGTGCCACG C A T	GCTGCAGATCTCGACGACGACCCTCTG A A D L D D D P L	TATTTTGGA Y F G	3.3	1	1.2	403992
	7.2	TGCGCCAGC C A S	AGTAGAGATCTAGTCGCAGAGACGCTG S R D L V A E T L	TACTTCGGC Y F G	2.4	23	44.2	403957
	7.2	TGCGCCAGC C A S	AGTACCGTGACAGCTCAGATCCAG S T V T A Q I Q	TACTTCGGG Y F G	3.5	12	23.1	403957
	10.2	TGTGCCAGC C A S	AGCCAGGCTGAAGTCTCTGGGGGGGCACCTTGGATCCCTCTG S Q A E V S G G A P W I P L	TATTTTGGA Y F G	3.3	1	1.9	403957
	12.1	TGCGCCAGC C A S	AGTTACGGGGGGAGCGGTTCCTATGAGCAG S Y G G S G S Y E Q	TATTTCGGC Y F G	3.7	16	30.8	403957

(Table continues)

(Continued Table)

Epitope	Sequence					Number of sequence	Frequency (%)	Animal
	Vβ	FR	CDR3β	FR	Jβ			
$Tp2_{50-59}$	1.8	TGTGCCAGC C A S	AGCCAGGATCCAGAGACGCTG S Q D P E T L		TACTTCGGG Y F G	2.4	25	28.7	302186
	2.7	TGTGGTGCT C G A	AGAGGATTAGGGGAGGTT R G L G E V		TTCTTTGGA F F G	1.1	3	3.4	302186
	28	TGTGCCAGC C A S	GCTGAATATGGGGGGGAGAACACCCAGCCCCTG A E Y G G E N T Q P L		TACTTTGGA Y F G	3.2	58	***66.7***	302186
	28	TGTGCCAGC C A S	GCTGAATATGGGGGGAAGAACACCCAGCCCCTG A E Y G G K N T Q P L		TACTTTGGA Y F G	3.2	1	1.1	302186
	1.3	TGTGCCAGC C A S	AGCCCGCTTGGGGGGTATCGAAGCGCCGTGCAGCTG S P L G G Y R S A V Q L		TACTTTGGA Y F G	2.2	1	1.2	403992
	5	TGTGCCAGC C A S	GTCTCGCCTGGTGGGGACTAT V S P G G D Y		CACTTCGGC H F G	1.2	83	98.8	403992
	7.2	TGCGCCAGC C A S	AGTAGAGATCTAGTCGCAGAGACGCTG S R D L V A E T L		TACTTCGGC Y F G	2.4	5	7.9	403957
	12.1	TGCGCCCAG C A Q	CATTCTTCGCGGGAGCAG H S S R E Q		TATTTCGGC Y F G	3.7	58	92.1	403957
$Tp2_{98-106}$	13.5	TGTACCAGC C T S	AATTTGGGGGGCCTGGACCTGGGCACTCAG N L G G L D L G T Q		TACTTCGGC Y F G	2.3	72	98.6	302186
	13.5	TGTACCAGC C T S	ACATTTGGGGGGTGGGCCCTCGAGACGCTG T F G G W A L E T L		TACTTCGGG Y F G	2.4	1	1.4	302186
	4.3	TGCAGTGCT C S A	GGTTCGGGCTATGAGCAG G S G Y E Q		TATTTCGGc Y F G	3.7	42	71.2	403992
	14	TGTGCCAGC C A S	AGTCCGGGACTGGGGGTTCAGATCCAG S P G L G V Q I Q		TACTTCGGG Y F G	3.5	12	20.3	403992
	15.2	TGTGCCAGC C A S	AATGCAGGGCAGCAGGGGGGCACCCAGCCCCTG N A G Q Q G G T Q P L		TACTTTGGA Y F G	3.2	5	8.5	403992

(Table continues)

(Continued Table)

Epitope	Sequence					Number of sequence	Frequency (%)	Animal
	Vβ	FR	CDR3β	FR	BJ			
$Tp2_{98-106}$	1	TGTGCCAGC C A S	AGTGTTCGGGGGGGAGACACGCAG S V R G G D T Q	TACTTCGGC Y F G	2.3	1	1.2	403957
	1.7	TGTGCCAGC C A S	AGCCAAGATTATGGCGCGACGCTG S Q D Y G A T L	TACTTCGGG Y F G	2.4	1	1.2	403957
	12.1	TGCGCCACG C A T	CTAATTCTTGCGCGGGAGCAG L I L A R E Q	TATTTCGGC Y F G	3.7	1	1.2	403957
	13.5	TGTACCAGC C T S	AGTCGGGGGGGGCGAATAGATGGGGAGCTG S R G G R I D G E L	CACTTCGGG H F G	3.1	3	3.7	403957
	13.5	TGTACCAGC C T S	AGTCCGGGGGGGGGCGAATAGATGGGGAGCTG S P G G G R I D G E L	CACTTCGGG H F G	3.1	1	1.2	403957
	13.5	TGTACCAGC C T S	ACATTGGGGGGGATCGTCTATGAGCAG T L G G I V Y E Q	TATTTCGGC Y F G	3.7	68	82.9	403957
	14	TGTGCCAGC C A S	AGTGAACGGTTCGGGGGGGATGGGAACAACCCTCTG S E R F G G D G N N P L	TATTTTGGA Y F G	3.3	6	7.3	403957
	14	TGTGCCAGC C A S	AGTCCCCAGTTCGGGGGGGGGCATCCCACTCAGATCCAG S P Q F G G G H P T Q I Q	TACTTCGGG Y F G	3.5	1	1.2	403957

B) Sequences of $Tp2_{98-106}$ epitope-specific TRB clonotypes from three A10-heterozygous cattle

Epitope	Sequence					Number of sequence	Frequency (%)	Animal
	Vβ	FR	CDR3β	FR	BJ			
$Tp2_{98-106}$	1.5	TGTGCCAGC C A S	AGCGAATATCCTCGGGGGGGGCATAGCAACCCTCTG S E Y P R G G H S N P L	TATTTTGGA Y F G	3.3	2	2.7	102121
	4.3	TGCAGTGCT C S A	GGATCATGGGAGTCAGAGACGCTG G S W E S E T L	TACTTCGGG Y F G	2.4	1	1.4	102121
	7	TGCGCCAGC C A S	AGCTCGGGCTTCGGGGGTGATGACACGCAG S S G F G G D D T Q	TACTTCGGC Y F G	3.4	13	17.6	102121
	13.5	TGTACCAGC C T S	AGTTTGGGGGGTCCGTACTCAGAGACGCTG S L G G P Y S E T L	TACTTCGGG Y F G	2.4	57	77.0	102121
	20.1	TGTGCCTGG C A W	ACTTCGGGGGGATGGAACAACCCTCTG T S G G W N N P L	TATTTTGGA Y F G	3.3	1	1.4	102121
	13.5	TGTACCAGC C T S	AGTCTCAGCGGGGAGACGCTG S L S G E T L	TACTTCGGG Y F G	2.4	2	2.3	102170
	13.5	TGTACCAGC C T S	AACCTGGGGGGCATCACAGACACGCAG N L G G I T D T Q	TACTTCGGC Y F G	3.4	84	97.7	102170
	4.3	TGCAGTGCT C S A	CCCGGGACCGAGGGCTATGAGCAG P G T E G Y E Q	TATTTCGGC Y F G	3.7	24	28.2	402082
	4.6	TGCAGTGCC C S A	GGGGGGGACAGCTATGAGCAG G G D S Y E Q	TATTTCGGC Y F G	3.7	12	14.1	402082
	13.5	TGTACCAGC C T S	AGTTTGGACAGCCTCCGGGGCTAT S L D S L R G Y	CACTTCGGC H F G	1.2	10	11.8	402082
	13.5	TGTACCAGC C T S	AGTCAGGACCCGCATTCGGGAGGGAACACCCAGCCCCTG S Q D P H S G G N T Q P L	TACTTTGGA Y F G	3.2	39	45.9	402082

Appendix C. A typical example of PCR and sequencing error correction for clonotypes extracted from Illumina sequencing reads

CDR3β sequences defined through identification of the three nucleotides encoding the conserved amino acid Cys (C) located towards the 3' end of the V gene segment and the three nucleotides encoding the conserved amino acid Phe (F) in 5' end of the J gene segment were used for PCR and sequencing error correction, in order to define the real clonotype diversity. Low-abundant clonotypes with PCR and/or sequencing errors that can be safely corrected are shown in Table C-1; those clonotypes with nucleotide variants in gene junctions and clonotypes with more than 3 errors are shown in Table C-2. Number of sequencing reads, CDR3β amino acid sequences and CDR3β nucleotide sequences are shown in the first, second and third column. Vβ, Jβ and identifiable Dβ germline gene components of CDR3β are shown in the last three columns. The dominant "core clonotype", which determines the nucleotide sequence of the final clonotype, is shown in bold with identified Vβ (orange), Dβ (blue) and Jβ (green) gene components. Corrected errors (red font and bold) within CDR3β sequences are shown. Errors at gene junctions are shown in purple font and are underlined.

Table C-1 Error correction to merge low-abundant clonotypes into a dominant clonotype

Number of sequencing reads	CDR3β, amino acid sequences	CDR3β, nucleotide sequences	Vβ	Jβ	Dβ
3343	**CASDPSGGANTQPLYF**	**TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT**	**28**	**3.2**	**3**
25	CASDPSGGANAQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACGCCCAGCCCCTGTACTTT	28	3.2	3
21	CASDPAGGANTQPLYF	TGTGCCAGCGACCCTGCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
17	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCGTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
10	CASDPSGGANPQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACCCCCAGCCCCTGTACTTT	28	3.2	3
6	CASDPTGGANTQPLYF	TGTGCCAGCGACCCTACGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
5	CASDPSGGASTQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAGCACCCAGCCCCTGTACTTT	28	3.2	3
5	CASDPSGGAITQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGATCACCCAGCCCCTGTACTTT	28	3.2	3
5	CASDPSGGANTQPLDF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGGACTTT	28	3.2	3
4	CASDPSAGANTQPLYF	TGTGCCAGCGACCCTTCGGCGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
4	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCCTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
2	CVSDPSGGANTQPLYF	TGTGTCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
2	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCTGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
2	CASDPSVGANTQPLYF	TGTGCCAGCGACCCTTCGGTGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
2	CASDPSGGANTQPLDF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGGACTTT	28	3.2	3
2	CASDPLGGANTQPLYF	TGTGCCAGCGACCCTTTGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3

2	CASDPSGGANTQPLCF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGTGCTTT	28	3.2	3

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Number of sequencing reads	CDR3, amino acid sequences	CDR3, nucleotide sequences	Vβ	Jβ	Dβ
2	CASDPSGGANTQPRYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCGGTACTTT	28	3.2	3
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCGCTGTACTTT	28	3.2	3
1	CASDPSGGAKTQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAAAACCCAGCCCCTGTACTTT	28	3.2	3
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCACTGTACTTT	28	3.2	3
1	CASDPSGGAKTQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAAGACCCAGCCCCTGTACTTT	28	3.2	3
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAACCCCTGTACTTT	28	3.2	3
1	CASDPSGGANTQPL*F	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGTAGTTT	28	3.2	3
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACTCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGANTEPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCGAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGANT*PLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCTAGCCCCTGTACTTT	28	3.2	3
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCGGGGGGAGCGAATACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGATTQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGACCACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPPGGASTQPLYF	TGTGCCAGCGACCCTCCGGGGGGAGCGAGCACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGANTQHLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCACCTGTACTTT	28	3.2	3
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTCTACTTT	28	3.2	3
1	CDSDPSGGANTQPLYF	TGTGACAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3

1	CASDPSGGANTQP**P**YF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCC**C**GTACTTT	28	3.2	3
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCC**G**CTGTACTTT	28	3.2	3

(Page 2 of 5 pages for Table C-1)

Number of sequencing reads	**CDR3, amino acid sequences**	**CDR3, nucleotide sequences**	**Vβ**	**Jβ**	**Dβ**
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCGGG**T**GGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCT**T**TACTTT	28	3.2	3
1	CASDPS**R**GANTQPLYF	TGTGCCAGCGACCCTTCG**C**GGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGAN**I**QPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACA**T**CCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGANT**K**PLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACC**A**AGCCCCTGTACTTT	28	3.2	3
1	C**S**SDPSGGANTQPLYF	TGT**T**CCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCC**A**TCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	**R**ASDPSGGANTQPLYF	**C**GTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCC**A**CTGTACTTT	28	3.2	3
1	CASDPS**E**GANTQPLYF	TGTGCCAGCGACCCTTCGG**A**GGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPS**R**GANTQPLYF	TGTGCCAGCGACCCTTCG**A**GGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTC**C**GGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCA**A**CCCCTGTACTTT	28	3.2	3
1	CASDPSGGANTQPL***F	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGTA**G**TTT	28	3.2	3
1	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCGGGGGGAGCGAACAC**G**CAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGANTQPL**F**F	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGT**T**CTTT	28	3.2	3
1	CASDPSG**R**ANTQPLYF	TGTGCCAGCGACCCTTCGGGG**C**GAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3

1	CASDP*GGANTQPLYF	TGTGCCAGCGACCCTTAGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3

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Number of sequencing reads	CDR3, amino acid sequences	CDR3, nucleotide sequences	Vβ	Jβ	Dβ
1	WASDPSGGANTQPLYF	TGGGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGANTHPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCACCCCCTGTACTTT	28	3.2	3
1	CASDPSGGAITQPLYF	TGTGCCAGCGACCCCTCGGGGGGAGCGATCACCCAGCCCCTGTACTTT	28	3.2	3
1	CSSDPSGGANTQPLYF	TGTTCCAGCGACCCGTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
2	CASDPAGGANAQPLYF	TGTGCCAGCGACCCTGCGGGGGGAGCGAACGCCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSG*ANTQPLYF	TGTGCCAGCGACCCTTCGGGGTGAGCGAACACCCAGCCCCTATACTTT	28	3.2	3
1	CASDPSAGAITQPLYF	TGTGCCAGCGACCCTTCGGCGGGAGCGATCACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSVGATTQPLYF	TGTGCCAGCGACCCTTCGGTGGGAGCGACCACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGANSQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACTCCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGATAQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGACCGCCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGANTQLLSF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCTCCTGTCCTTT	28	3.2	3
1	CASDPSGGANAQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAATGCCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGAIPQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGATCCCCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGANSQPLCF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACTCCCAGCCCCTGTGCTTT	28	3.2	3
1	CASDPSGGATTQPLFF	TGTGCCAGCGACCCTTCGGGGGGAGCGACCACCCAGCCCCTGTTCTTT	28	3.2	3
1	CASDPSGGAIAQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGATCGCCCAGCCCCTGTACTTT	28	3.2	3

1	CASDPSGGANAEPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAACGCCGAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGAITQPLSF	TGTGCCAGCGACCCTTCGGGGGGAGCGATCACCCAGCCCCTGTCCTTT	28	3.2	3

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Number of sequencing reads	CDR3, amino acid sequences	CDR3, nucleotide sequences	Vβ	Jβ	Dβ
1	CASDPSGGASAQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGAGCGCCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSVGATTQPLSF	TGTGCCAGCGACCCTTCGGTGGGAGCGACCACCCAGCCCCTGTCCTTT	28	3.2	3
1	CASDPSEGANAQPLYF	TGTGCCAGCGACCCTTCGGAGGGAGCGAATGCCCAGCCCCTGTACTTT	28	3.2	3

(Page 5 of 5 pages for Table C-1)

Table C-2 Clonotypes with nucleotide variants in gene junctions and clonotypes with more than 3 errors in CDR3β

Number of sequencing reads	CDR3, amino acid sequences	CDR3, nucleotide sequences	Vβ	Jβ	Dβ
3343	**CASDPSGGANTQPLYF**	TGTGCCAGCGACCCTTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
9	CASDRSGGANTQPLYF	TGTGCCAGCGACCGTTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
3	CASDPSGVANTQPLYF	TGTGCCAGCGACCCTTCGGGGGTAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
2	CASDPSGAANTQPLYF	TGTGCCAGCGACCCTTCGGGGGCAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
2	CASDHSGGANTQPLYF	TGTGCCAGCGACCATTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
2	CASDPSGGADTQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGGACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGAHTQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGCACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGRANTQPLYF	TGTGCCAGCGACCCTTCGGGGCGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGEANTQPLYF	TGTGCCAGCGACCCTTCGGGGGAAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGAETQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGGAGACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGGACTQPLYF	TGTGCCAGCGACCCTTCGGGGGGAGCGTGCACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDRSGGANAQPLYF	TGTGCCAGCGACCGTTCGGGGGGAGCGAACGCCCAGCCCCTGTACTTT	28	3.2	3
1	CTSDPSGGADTQPLYF	TGTACCAGCGACCCTTCGGGGGGAGCGGACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSWGADTQPLYF	TGTGCCAGCGACCCTTCGTGGGGAGCGGACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGRANAQPLYF	TGTGCCAGCGACCCTTCGGGGCGAGCGAACGCCCAGCCCCTGTACTTT	28	3.2	3

1	CASDRSGGADPQPLYF	TGTGCCAGCGACCGTTCGGGGGGAGCGGACCCCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPAGGADTQPLYF	TGTGCCAGCGACCCTGCGGGGGGAGCGGACACACAGCCCCTGTACTTT	28	3.2	3

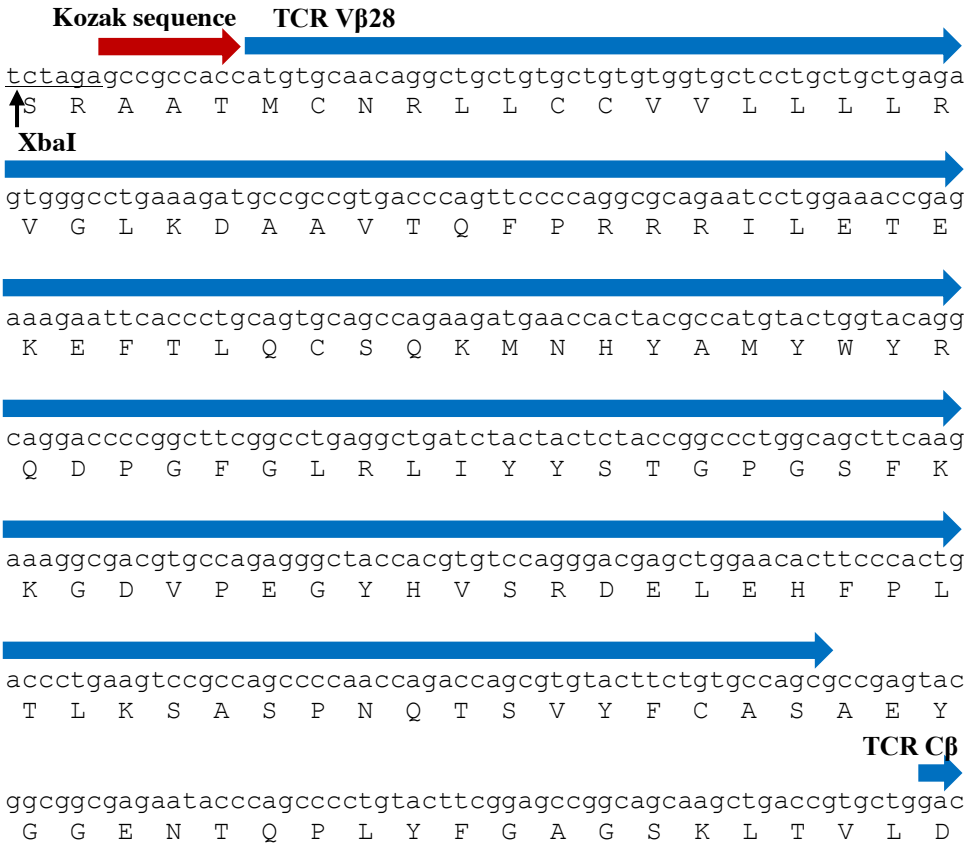
(Page 1 of 2 pages for Table C-2)

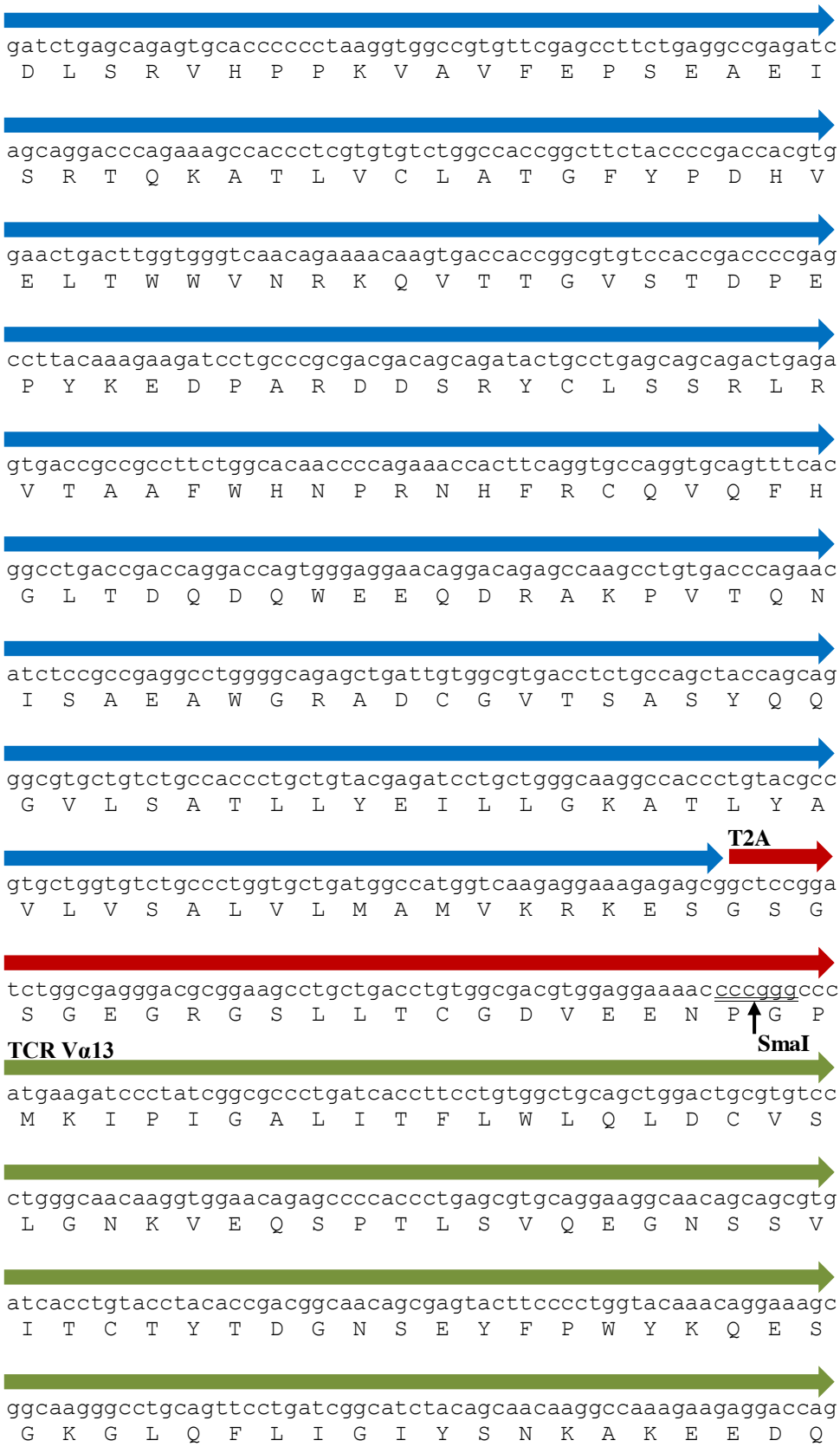
Number of sequencing reads	CDR3, amino acid sequences	CDR3, nucleotide sequences	Vβ	Jβ	Dβ
1	CASDRSEGANA*PLYF	TGTGCCAGCGACCGTTCGGAGGGAGCGAACGCCTAGCCCCTGTACTTT	28	3.2	3
1	CASDRAGGAIAQPLYF	TGTGCCAGCGACCGTGCGGGGGGAGCGATCGCCCAGCCCCTGTACTTT	28	3.2	3
1	CASDRAGGAVTQPLYF	TGTGCCAGCGACCGTGCGGGGGGAGCGGTCACGCAGCCCCTGTACTTT	28	3.2	3
1	CASDPAGGAYAQPLYF	TGTGCCAGCGACCCTGCGGGGGGAGCGTACGCACAGCCCCTGTACTTT	28	3.2	3
1	CASDLSGGANTQPLYF	TGTGCCAGCGACCTGTCGGGGGGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGRANTQPLYF	TGTGCCAGCGACCCGTCGGGGCGAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPSGAANTQPLYF	TGTGCCAGCGACCCGTCGGGGGCAGCGAACACCCAGCCCCTGTACTTT	28	3.2	3
1	CASDPAVGANAQPLYF	TGTGCCAGCGACCCTGCGGTGGGAGCGAACGCCCAGCCGCTGTACTTT	28	3.2	3
1	CASDPSWGASAQPLYF	TGTGCCAGCGACCCTTCGTGGGGAGCGAGCGCCCAGCCGCTGTACTTT	28	3.2	3
1	CASDRAEAANTQPLYF	TGTGCCAGCGACCGTGCGGAGGCAGCGAACACACAGCCCCTGTACTTT	28	3.2	3

(Page 1 of 2 pages for Table C-2)

Appendix D. Designed and synthesized bovine TCRβ-2A-TCRα constructs

Full-length cDNA of selected bovine TCR α and β chains were obtained from the $Tp2_{50-59}$ epitope-specific CD8 T cell clones, according to section 2.3.4. cDNAs of TCRβ28 (Vβ28-CASAEYGGENTQPL-Jβ3.2) and TCRα13 (Vα13-CAASDRSNYKLTF-Jα20) were cloned and sequenced, and initially used to generate the TCRβ28-2A-TCRα13 construct. Sequence of the synthesized construct is shown in Figure D.1. In order to generate other constructs, full-length cDNA of TCRα8 (Vα8.4-CALLRSGWQLTF-Jα8.2) and TCRα26 (Vα26-CILMAGYQKLTF-Jα8.2) were obtained and synthesized, as shown in Figure D.2. Restriction sites (SmaI and BamHI) in the synthesized genes allow gene segments replacement to obtain new TCRβ-2A-TCRα constructs. All constructs generated have the same TCRβ28 chain with different TCRα chains.




gatctgagcagagtgcacccccctaaggtggccgtgttcgagccttctgaggccgagatc
D L S R V H P P K V A V F E P S E A E I
agcaggacccagaaagccaccctcgtgtgtctggccaccggcttctaccccgaccacgtg
S R T Q K A T L V C L A T G F Y P D H V
gaactgacttggtgggtcaacagaaaacaagtgaccaccggcgtgtccaccgaccccgag
E L T W W V N R K Q V T T G V S T D P E
ccttacaaagaagatcctgcccgcgacgacagcagatactgcctgagcagcagactgaga
P Y K E D P A R D D S R Y C L S S R L R
gtgaccgccgccttctggcacaaccccagaaaccacttcaggtgccaggtgcagtttcac
V T A A F W H N P R N H F R C Q V Q F H
ggcctgaccgaccaggaccagtgggaggaacaggacagagccaagcctgtgacccagaac
G L T D Q D Q W E E Q D R A K P V T Q N
atctccgccgaggcctggggcagagctgattgtggcgtgacctctgccagctaccagcag
I S A E A W G R A D C G V T S A S Y Q Q
ggcgtgctgtctgccaccctgctgtacgagatcctgctgggcaaggccaccctgtacgcc
G V L S A T L L Y E I L L G K A T L Y A
T2A
gtgctggtgtctgccctggtgctgatggccatggtcaagaggaaagagagcggctccgga
V L V S A L V L M A M V K R K E S G S G
tctggcgagggacgcggaagcctgctgacctgtggcgacgtggaggaaaaccccgggccc
S G E G R G S L L T C G D V E E N P G P
SmaI
TCR Vα13
atgaagatccctatcggcgccctgatcaccttcctgtggctgcagctggactgcgtgtcc
M K I P I G A L I T F L W L Q L D C V S
ctgggcaacaaggtggaacagagccccaccctgagcgtgcaggaaggcaacagcagcgtg
L G N K V E Q S P T L S V Q E G N S S V
atcacctgtacctacaccgacggcaacagcgagtacttcccctggtacaaacaggaaagc
I T C T Y T D G N S E Y F P W Y K Q E S
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G K G L Q F L I G I Y S N K A K E E D Q

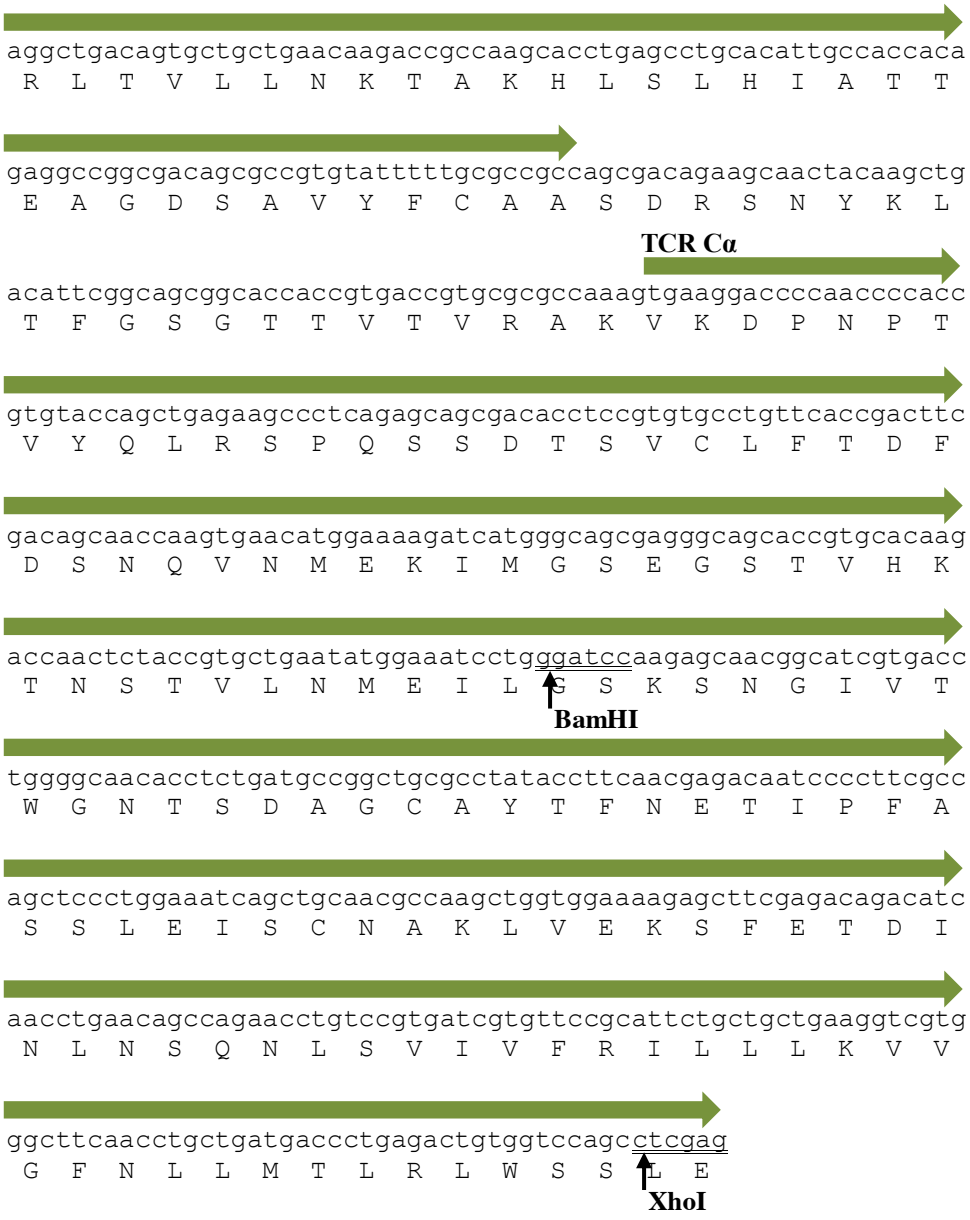
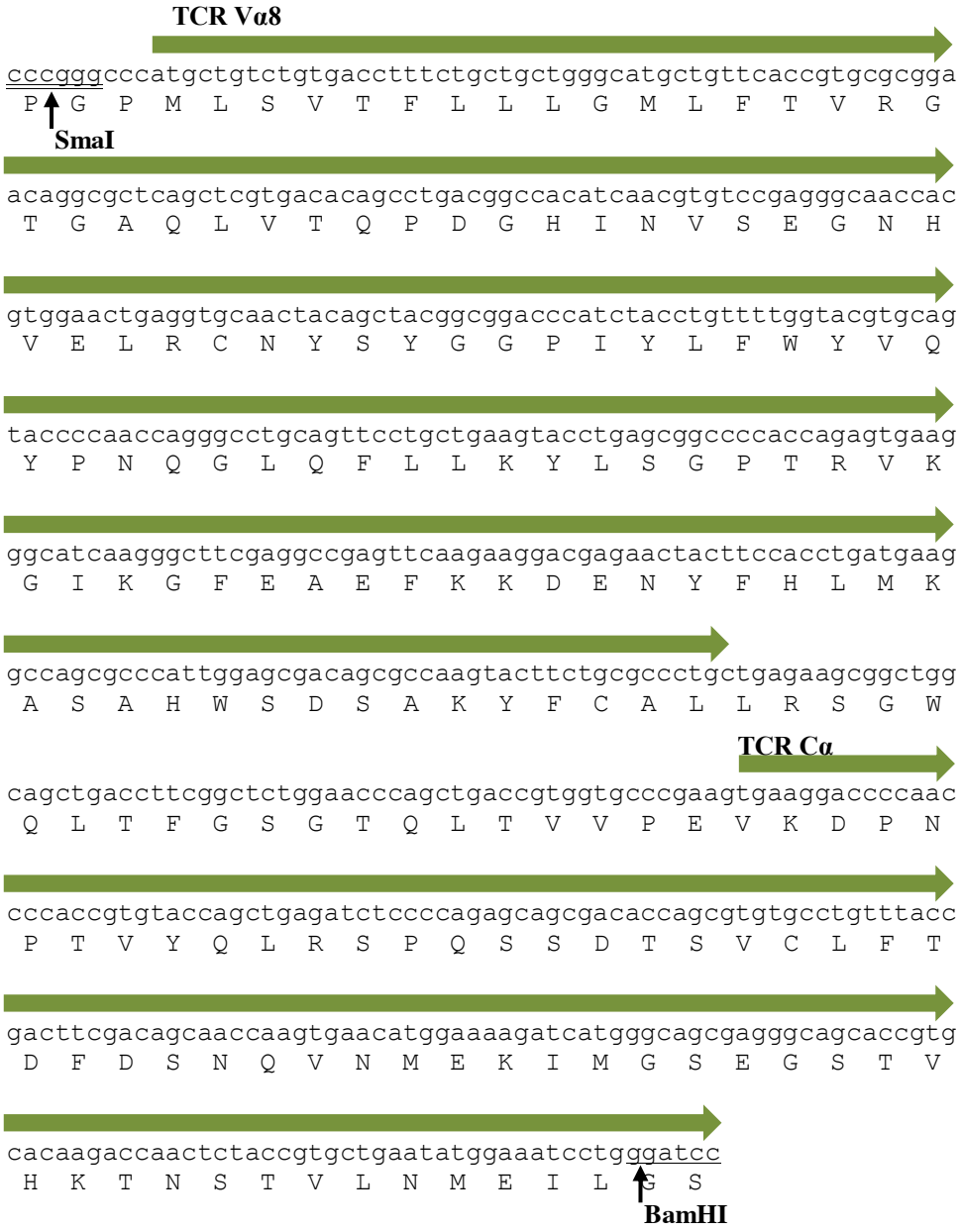


Figure D.1 Sequence of the synthesized bovine TCRβ-2A-TCRα construct. Restriction sites XbaI (tctaga) and XhoI (ctcgag) were added to the 5' and 3' ends of the construct for reconstitution of lentivirus-TCR vector. Kozak sequence (gccgccacc) at the 5' end of the construct is for the initiation of gene translation in cultured cells. TCR β and α gene segments are labelled. A self-cleaving sequence T2A connects the TCR β and α chains.


TCR Vα8
cccgggcccatgctgtctgtgacctttctgctgctgggcatgctgttcaccgtgcgcgga
P G P M L S V T F L L L G M L F T V R G
SmaI
acaggcgctcagctcgtgacacagcctgacggccacatcaacgtgtccgagggcaaccac
T G A Q L V T Q P D G H I N V S E G N H
gtggaactgaggtgcaactacagctacggcggacccatctacctgttttggtacgtgcag
V E L R C N Y S Y G G P I Y L F W Y V Q
taccccaaccagggcctgcagttcctgctgaagtacctgagcggccccaccagagtgaag
Y P N Q G L Q F L L K Y L S G P T R V K
ggcatcaagggcttcgaggccgagttcaagaaggacgagaactacttccacctgatgaag
G I K G F E A E F K K D E N Y F H L M K
gccagcgcccattggagcgacagcgccaagtacttctgcgccctgctgagaagcggctgg
A S A H W S D S A K Y F C A L L R S G W
TCR Cα
cagctgaccttcggctctggaacccagctgaccgtggtgcccgaagtgaaggaccccaac
Q L T F G S G T Q L T V V P E V K D P N
cccaccgtgtaccagctgagatctccccagagcagcgacaccagcgtgtgcctgtttacc
P T V Y Q L R S P Q S S D T S V C L F T
gacttcgacagcaaccaagtgaacatggaaaagatcatgggcagcgagggcagcaccgtg
D F D S N Q V N M E K I M G S E G S T V
cacaagaccaactctaccgtgctgaatatggaaatcctgggatcc
H K T N S T V L N M E I L G S
BamHI

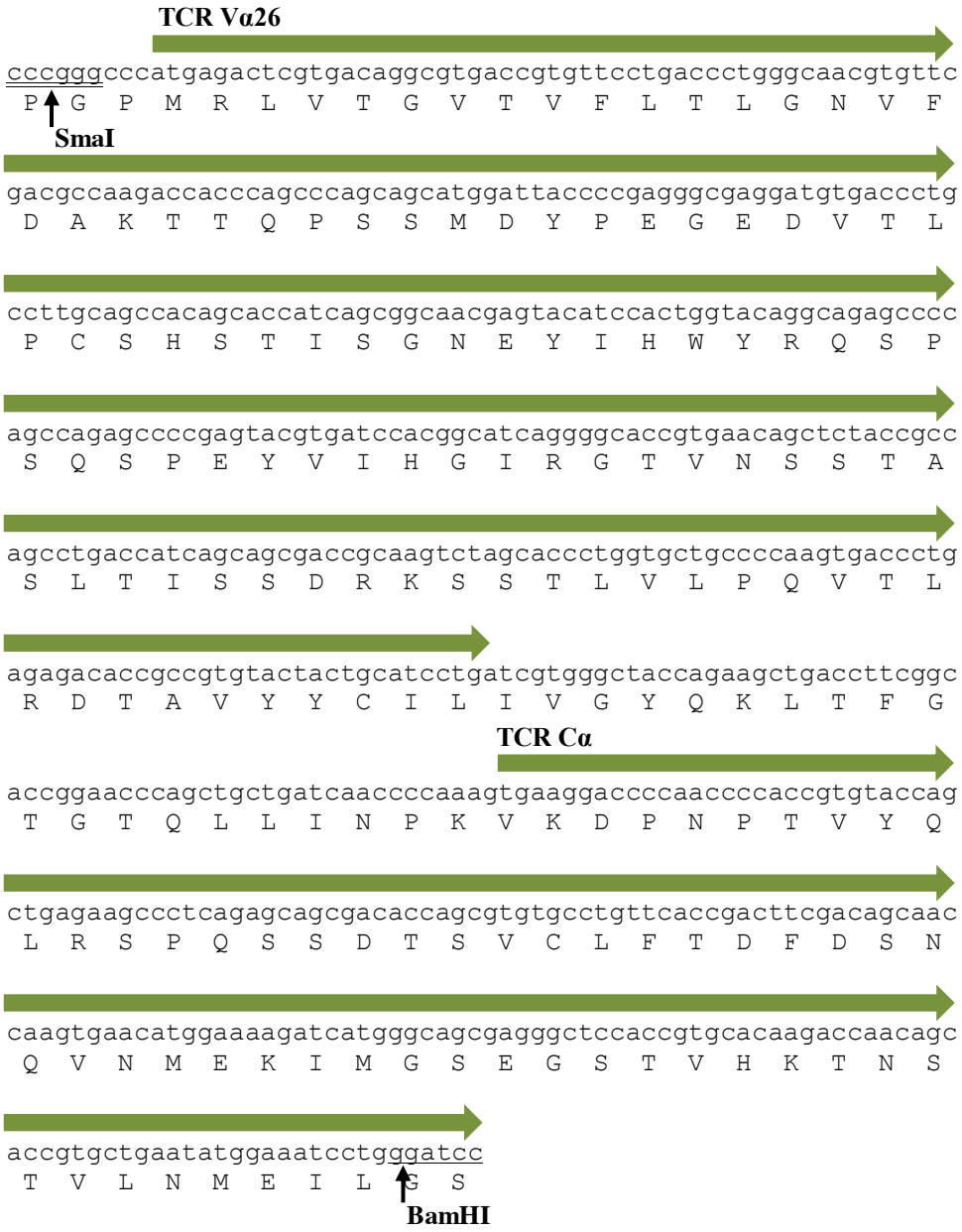


Figure D.2 Sequences of synthesized TCRα8 and TCRα26 genes. With the restriction sites SmaI (cccggg) and BamHI (ggatcc), the synthesized genes are used to replace the TCRα13 in the initially synthesized TCRβ-2A-TCRα construct. The resultant constructs have the same TCRβ28 gene with different TCRα genes.

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